THE
BIOLOGICAL BULLETIN

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THE

BIOLOGICAL BULLETIN

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HYPOPHYSIAL CONTROL OF CUTANEOUS PIGMENTATION IN AN ELASMOBRANCH FISH [1]

HELEN M. LUNDSTROM AND PHILIP BARD

(From the Marine Biological Laboratory, Woods Hole)

In a study of the effects of ablation of various parts of the brain of the dogfish, *Mustelis canis*, it was noticed that certain operated individuals became, in the course of the first few post-operative hours, lighter in color than their fellows. The degree of paling was such as to render the affected animal distinctly lighter than any normal dogfish of this species that we have seen. Inspection of the cranial contents of the operated fish showed that whenever the albinous appearance was produced the pituitary body had been extirpated in the intracranial intervention. On the other hand, the hypophysis was invariably intact in those operated fish which retained their normal dark grayish color. Quite naturally we were led to suspect that the dogfish possesses an hypophysial control of its cutaneous pigmentation similar to that which has been so clearly established for the amphibia by P. E. Smith (1916), Allen (1917, 1920), Atwell (1919), Rehberg and Krogh (Krogh, 1922) and Hogben and his collaborators (Hogben, 1924). It is a curious fact that, so far as we have been able to ascertain, no experimental investigation of chromatic function in the elasmobranch group has ever been made, although these animals possess dermal chromatophores (melanophores and xanthophores) not unlike those seen in the frog (Daniel, 1922).

That pituitary removal alone is followed by the development of pallor was shown in a series of hypophysectomies performed on 40 dogfish. The operation is easily carried out through a buccal approach.

[1] A report of results obtained in an investigation carried out in the Course in Physiology at the Marine Biological Laboratory, Woods Hole, during the summer of 1931.

Method

The fish was tied down in the dorsal position on a small shark board and a stream of sea water introduced by means of a tube into the pharyngeal region to maintain respiration (under these circumstances normal breathing movements continue). After incision and retraction of the mucous membrane the base of the skull was lightly scraped along the midline at the level of the pupils of the eyes. This brings to view through the translucent cartilage the chiasma, hypothalami, and posterior hypophysis. A medial opening was then cut in the skull between the chiasma and the posterior lobe. This exposes the elongated anterior lobe of the pituitary which lies in the depression between the hypothalami and extends forward nearly to the chiasma. The width of the exposure was such as to lay bare the medial half of each ventral hypothalamic surface. So much may be done without the slightest hemorrhage. But it was found that extension of the skull defect backward so as to expose merely the cranial aspect of the large bifid neuro-intermediate or posterior lobe resulted in profuse bleeding. Therefore this structure was carefully scooped out by means of a small curette introduced through the limited opening. With a little care this can be done without provoking hemorrhage and with minimal injury to the base of the brain. The anterior lobe may be left intact or it may be picked up with forceps, separated from the overlying nervous tissue, and so extirpated with very little damage to the hypothalamus. No attempt was made to close the wound. Provided the base of the brain had not been severely traumatized, the operated fish swam around normally and showed a general behavior indistinguishable from that of unoperated specimens. They usually succumbed after three or four days with loss of righting reactions and dyspnea as premonitory symptoms. A few lived as long as a week, which is about the average length of life of unoperated specimens kept under laboratory conditions. It seems likely that death was often induced by cerebral injury, some degree of cerebral herniation being the usual finding at autopsy. We have not been disturbed by this rather crude postoperative situation. It is apparently without significance for the specific problem in hand, namely, the experimental analysis of pituitary control of cutaneous pigmentation, for injury or herniation of the hypothalamic region without removal of the hypophysis never gave rise to the slightest pallor. Several animals survived such a condition several days and retained the same shade as normal control animals.

Results

Complete removal of the pituitary body (30 experiments) always resulted in pallor. Slight paling was first noticeable about thirty minutes after pituitary ablation and after three hours the change was generally very marked, but maximal pallor was not attained until about the twelfth postoperative hour. It was our practice to select pairs of fish having the same degree of pigmentation, hypophysectomize one and retain the other for comparison. Plate 1 is a photograph of two such fish taken twenty-four hours after complete hypophysectomy in one. It shows clearly the extreme pallor characteristic of the animal without hypophysis. That the pallor is due to "contraction" of the dermal melanophores is shown in Plates 3 and 4, which are microphotographs of homologous pieces of skin taken from the same fish just before and nineteen hours after hypophysectomy. As shown in Plate 3 the melanophores of the normal fish are in a state of considerable expansion, while Plate 4 indicates clearly that the pallor developing after pituitary removal is the result of the assumption by the melanophores of a more or less rounded contour. Were it not for the outlines of several placoid scales one might easily mistake these plates for microphotographs of the skin of a normal and a hypophysectomized frog or axolotl.

Removal of the anterior lobe alone was done in five dogfish and these survived the operation for several days without showing the slightest change in cutaneous pigmentation. Nor did leaving the anterior lobe intact impede in any way the development of the pallor ensuing upon removal of the posterior lobe. As already mentioned, severe traumatization of the hypothalamus was found to be without influence on pigmentation provided the posterior lobe of the pituitary was not ablated. In four fish the anterior lobe and hypothalamic protuberances were exposed in the usual way, transverse incisions made in these structures to a depth of one or two millimeters and much of the substance of the hypothalamus pulled out with forceps. Two of these animals survived thirty hours without at any time presenting the slightest change in hue. The other two, whose color also remained unchanged, were subjected to posterior pituitary removal after twenty-four hours whereupon there ensued a pallor which developed at the usual rate and to the usual extent.

Further evidence for an hypophysial control of the cutaneous melanophores was sought in a study of the effects of pituitary preparations when injected into pale hypophysectomized dogfish. Each of five pale specimens received subcutaneously a crushed dogfish posterior lobe suspended in one or two cubic centimeters of sea water. A

distinct general darkening appeared within the first three minutes and after an hour these fish were indistinguishable from the darkest normal animals. Then in the course of the next five or six hours they gradually returned to the albinous condition. Injections of similar suspensions of cerebellum, skeletal muscle, and pancreas from normal dogfish were entirely without effect. In three experiments it was found that administration of crushed anterior lobe resulted in a moderate darkening which was slower to develop and which disappeared more rapidly than the more intense darkening caused by an equal quantity of crushed posterior lobe. In one pair of pale fish injection into one of a crushed posterior lobe fragment of about half the size of the anterior lobe produced an immediate darkening which reached its maximum in one hour, while injection of the crushed anterior lobes of two fish into the other produced a moderate darkening which was slow to develop and had a very short duration. These results together with those of the ablation experiments indicate that the posterior lobe is the chief source of the melanophore-expanding substance or secretion. The activity of the anterior lobe material may well have been due to diffusion into it of active substance of posterior lobe origin. Shortage of fish prevented our exploring this question further.

Extracts of several posterior lobes were made. Immediately after its removal from a living dogfish the lobe was crushed, ground up, stirred and shaken in sea water, and the resulting suspension filtered. After appropriate dilution with sea water the clear filtrate was tested by injection of varying amounts into thoroughly pale hypophysectomized fish of the same size as the donor of the lobe. It was found that an amount of filtrate corresponding to one twenty-fifth of one posterior lobe was necessary to produce a noticeable darkening while it required from one-tenth to one-seventh of a filtrate to make such a fish as dark as normal controls. It is unlikely that such simple extracts as these represent the full potency of the glands. Hogben (1924) states that the amount of melanophore stimulant in the pituitary of one frog is sufficient to darken the skin of fifty-six animals of the same species.

The posterior pituitary preparations "pituitrin" (Parke, Davis & Co.) and "infundin" (Burroughs, Wellcome & Co.) produced darkening in pale pituitaryless fish when given subcutaneously or intramuscularly. Plate 2 is a photograph of the pair of fish shown in Plate 1. It was taken one hour after the hypophysectomized individual had received a subcutaneous injection of 0.4 cc. of "obstetrical pituitrin," an amount equivalent to four international oxytocic units. It can be seen that this dose had darkened the animal almost to its preoperative

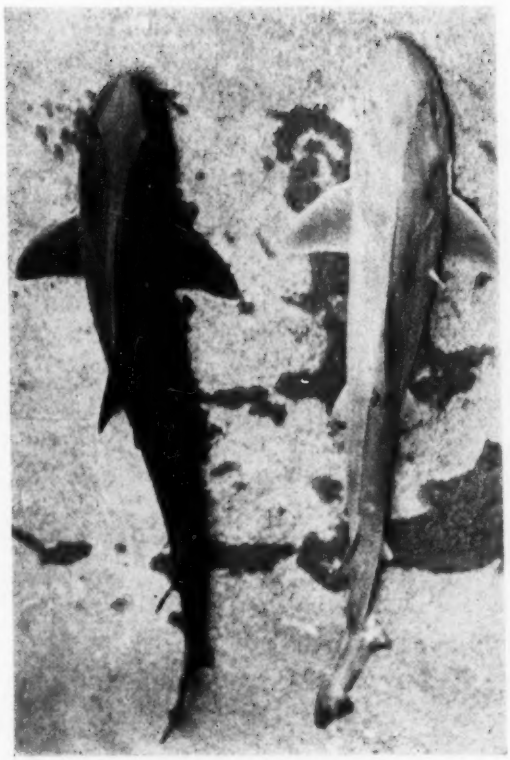

PLATE 1. Photograph of a pair of dogfish, originally of the same shade, taken twenty-four hours after removal of the hypophysis in the animal on the right. Both animals had been kept in the same tank and the operated individual showed no abnormalities of behavior.

PLATE 2. Photograph of the same pair of dogfish taken one hour after the hypophysectomized individual (on the right) had received a subcutaneous injection of 0.4 cc. of "obstetrical pituitrin" (Parke, Davis & Co.).

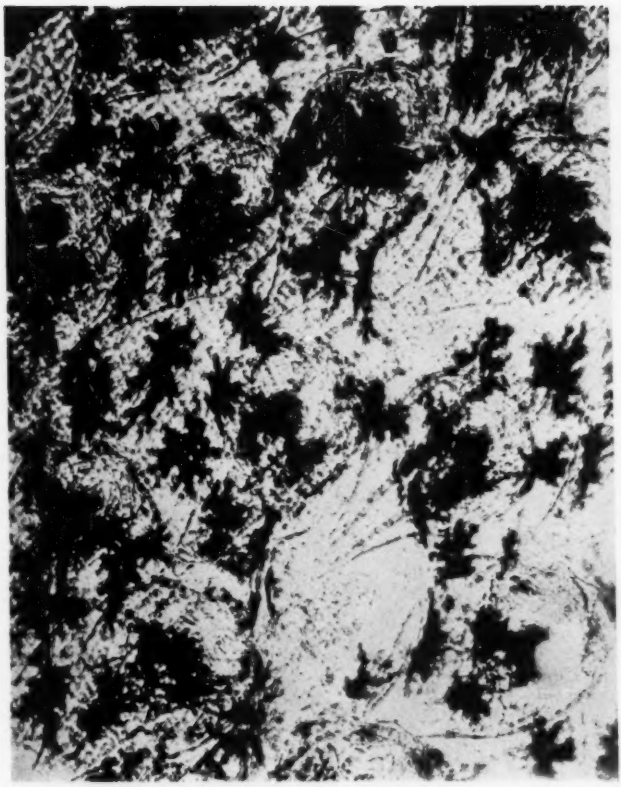

PLATE 3. Microphotograph of a mount of skin removed from a normal dogfish showing expanded condition of the melanophores.

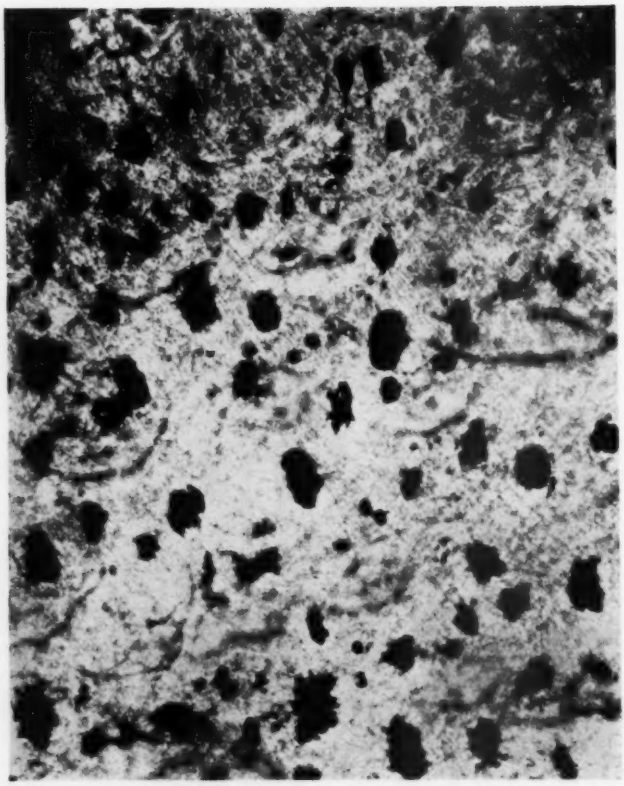

PLATE 4. Microphotograph of an homologous piece of skin taken from the same fish nineteen hours after hypophysectomy.

shade. In the case of several pale hypophysectomized fish, twenty to thirty inches in length, it was found that a subcutaneous dose of about 0.5 cc. (5 international units) of "obstetrical pituitrin" was required to produce in them a darkness equal to that of normal controls. It seemed interesting to test the action on elasmobranch melanophores of the two active principles separated from pituitary extracts by Kamm and his associates and now supplied by Parke, Davis and Co. under the names "pitressin" and "pitocin." It was found that 0.25 cc. (5 pressor units) of "pitressin" was capable of producing approximately the same effect as 0.5 cc. (5 international units) of "pituitrin." On the other hand 0.5 cc. (5 international units) of "pitocin" had no effect whatever on pale individuals of the same size. When twice this quantity of "pitocin" was injected there occurred a slight darkening which reached its maximum much more slowly and disappeared more rapidly than the reaction to "pitressin" or "pituitrin." Since "pitocin" contains one-half unit of pressor activity per cubic centimeter, it is probable that this mild positive result was due to the pressor substance present. These results are in general agreement with those of L. W. Rowe (1928), who reported that "vasopressin" ("pitressin") but not "oxytocin" ("pitocin") caused expansion of frog melanophores, a finding which is in contradiction to the inference of Hogben and Winton (1922*b*) that the uterine and melanophore stimulants of pituitary extracts are identical.

We had hoped to secure evidence of the presence of melanophore-expanding material in the circulating blood, but shortage of dogfish during the latter part of the summer made this impossible. Indirect evidence that pituitary substance acts directly on the dermal melanophores was, however, secured in our observation, often repeated, that the melanophores of small isolated pieces of skin from pale dogfish expanded markedly when such skin fragments were placed in dilute sea-water solutions of "pituitrin" or "pitressin" or in suspensions or extracts of dogfish posterior lobe. When such pieces were immersed in sea water alone the melanophores remained contracted. The expanded melanophores of bits of skin from normal fish gradually contract when placed in sea water. Addition of "pituitrin," etc., prevented this slow contraction. Equivalent solutions of "pitocin" had no action on the melanophores of skin fragments.

Discussion

The results presented in the foregoing section give abundant proof that the normal dark grayish color of the dogfish is maintained through the agency of the hypophysis. Apparently this organ continuously

delivers to the blood stream a quantity of melanophore stimulant capable of maintaining the rather widely expanded state of the cutaneous melanophores to which the normal color of the fish is due. The experimental findings strongly suggest that this humoral agent has its chief, if not its sole, origin in the posterior lobe. This portion of the gland is more correctly termed the neuro-intermediate lobe. Apart from a rather small extension of neuroglia fibers from the border of the infundibular cavity it is wholly composed of a highly vascular mass of basophil cells which, on the basis of embryological as well as histological observations, obviously constitutes the homologue of the pars intermedia of higher forms (de Beer, 1926). The ventral lobe of the selachian hypophysis which is attached dorsally to the neuro-intermediate lobe and ventrally to the skull and which, according to de Beer (1926), is composed largely but not entirely of basophil cells, has not been taken into separate account in our experiments. In all of our posterior lobe extirpations it was doubtless removed. It would seem highly probable that the melanophore-expanding substance originates in the pars intermedia. There is excellent evidence that such is the case in the amphibia. We know that there too anterior lobe removal is not followed by pallor (Hogben and Winton, 1923) and that while the melanophore stimulant is not lacking from extracts of pars anterior and pars nervosa of beef glands, there is more of it per gram of dried tissue in the pars intermedia than in the other two portions of the gland (Hogben and Winton, 1922*a*, 1922*b*). Some further confirmation of this point comes from Atwell's report (1919) that albinous tadpoles darken as a result of melanophore expansion when placed in dilute extracts of beef pars intermedia. But by far the most conclusive results have been those obtained by B. M. Allen (1920) in his study of the effects of transplantation of different parts of the hypophysis of the adult frog (*Rana pipiens*) into tadpoles from which the pars buccalis had been removed (anlage of pars nervosa left intact) and which, as a result, exhibited pallor as well as failure to metamorphose and retardation of growth. The operated larvae which received anterior lobe transplants showed "not the slightest tendency to return to the original black color except for a slight tendency at the beginning," although this treatment caused marked acceleration of growth. On the other hand, the albinous tadpoles regained their dark color when the intermediate lobe was engrafted into them. In a recent communication (1930) Allen reports experiments in confirmation of his earlier results and shows that transplants of pars nervosa do not cause pigmentary effects. Allen's work is a most significant contribution to the problem

of localization of pituitary function. Although it presents the only unequivocal evidence for the precise origin of the amphibian melanophore stimulant, it is not referred to in Hogben's monograph (1924). Shortly after the appearance of Allen's paper, Swingle (1921) reported that intraperitoneal transplantations of pars intermedia from various frogs into bullfrog larvæ caused darkening of the skin. It can be concluded that in the frog the pigmentary hormone is produced only by the pars intermedia and our own findings lend support to the view that such is the case in the elasmobranch.

It is well known that amphibia exhibit a rhythm of color response which depends on a balance of such environmental factors as humidity, temperature, oxygen supply, and illumination (Hogben, 1924). In the majority of amphibia light causes pallor while its absence promotes darkening. We have found that dogfish taken from a moderately illuminated tank paled markedly when placed in a brightly illuminated tank, while other fish originally of the same shade and from the same tank darkened perceptibly when placed in a tank from which nearly all light was excluded. These changes require several hours. They cannot, of course, be referred to changes in humidity and we were at some pains to be sure that during these tests the temperature and aëration of the tanks were equal. Segments of illuminated fish which were covered by bands of adhesive plaster did not remain dark. We conclude, therefore, that light causes pallor by a general rather than a local action, and since pale hypophysectomized fish remained pale when kept in darkened tanks we can assume that these pigmentary changes evoked by changes in illumination are the results of variations in pituitary activity.

Hogben (1924) is strongly of the opinion that regulation of color response in the amphibia by melanophore contraction and expansion is correlated with a fluctuating pituitary secretion. He believes this to be an adequate explanation of all the pigmentary phenomena seen in these animals and he is inclined completely to discount all assumptions of a direct nervous control of amphibian melanophores. He suggests that such chromatic effects as have been observed following nerve section or stimulation or drug administration have been due to vasomotor changes. Since it is clearly established that adrenalin injected intravenously causes pallor in the frog we made some effort to determine whether injection of this substance has any influence on the pigmentation of the dogfish. In a few preliminary experiments we gained the impression that large subcutaneous injections of adrenalin caused slight paling. Subsequently, at a time when the supply of fish had greatly diminished, we tested the effect of injections made

directly into the heart chambers. In collaboration with Dr. René Gayet, who kindly offered to share a number of fish with us, it was found that 1 cc. of a 1 : 1000 solution of adrenalin chloride evoked a paling which usually became noticeable after from five to ten minutes, sometimes not until after fifteen minutes, progressed to a maximum in about an hour, and persisted for approximately two hours. In one case the pallor was maximal; in the others it was not. Direct microscopical examination showed that in these cases the melanophores had actually undergone contraction. But the inadequate number of experiments performed makes it hazardous to venture an inference. Yet the comparative sluggishness of the responses provoked by these enormous doses which obviously produced a state of shock does not strongly suggest that the dogfish possesses an adrenal or sympathetic control. It may well have been that vasoconstriction of cutaneous vessels led to an asphyxiation and consequent contraction of the melanophores. Because of scarcity of material we were obliged to postpone to a later occasion an adequate investigation of the interesting question of a possible extra-pituitary control of elasmobranch chromatophores.

Summary

1. Complete hypophysectomy in the dogfish, *Mustelis canis*, results in pallor of the skin. This same result follows removal of the posterior (neuro-intermediate) lobe alone. Extirpation of the anterior lobe does not result in paling. Severe traumatization of the hypothalamus without destruction of the posterior lobe of the hypophysis has no influence on cutaneous pigmentation.

2. The pallor following hypophysectomy is due to "contraction" of the cutaneous melanophores which are normally in a state of considerable "expansion."

3. Suspensions or extracts of dogfish posterior lobe and the commercial posterior pituitary preparations, "pituitrin," "infundin," and "pitressin" produce melanophore expansion and consequent darkening when injected subcutaneously or intramuscularly into pale hypophysectomized dogfish. Suspensions of dogfish cerebellum, skeletal muscle, and pancreas have no effect upon pigmentation. The oxytocic principle of the preparation, "pitocin," does not produce melanophore expansion in hypophysectomized fish. Administration of suspensions of dogfish anterior lobe cause a relatively weak darkening of pale fish. The melanophores of isolated pieces of dogfish skin react to these various substances in the same way as do the melanophores of fish receiving them by injection.

4. Bright illumination of normal dogfish for several hours causes a

certain degree of pallor. Covered segments of the skin pale to the same extent as exposed regions. Absence of light leads to darkening of normal fish, but darkness does not modify the pallor of hypophysectomized individuals.

It is concluded that the posterior lobe of the hypophysis is responsible for the considerable degree of melanophore expansion characteristic of the skin of normal dogfish. Although the possibility of an extra-hypophysial control of cutaneous pigmentation has not been excluded, it is apparent that the pituitary gland plays a dominant rôle in the pigmentary alterations exhibited by the normal dogfish.

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REFLEX CARDIAC INHIBITION OF BRANCHIO-VASCULAR ORIGIN IN THE ELASMOBRANCH, SQUALUS ACANTHIAS

BRENTON R. LUTZ AND LELAND C. WYMAN

(From the Mount Desert Island Biological Laboratory and the Physiological Laboratory of Boston University School of Medicine)

Reflex cardiac and respiratory inhibition has been observed in elasmobranchs upon mechanical or electrical stimulation of various regions, external and internal, including the gill region (Lutz, 1930*a*). Reflex cardiac inhibition in mammals may also be obtained upon stimulating various regions. The distribution of the latter sensory areas is, however, much more restricted than in the elasmobranchs. Receptors which are especially important in regulating the circulation appear to be located in the vascular organs themselves. The depressor mechanism with receptors located in the aorta is well known (Eyster and Hooker, 1908; Anrep and Segall, 1926), and recently the carotid sinus has been shown to be an important zone, reflexly controlling heart rate and vasomotor tonus (Heymans, 1929). The physiological stimulus is apparently an alteration of pressure within this vessel.

The present paper is concerned with an attempt to ascertain whether similar alterations of pressure within the gill vessels of elasmobranchs are effective cardio-inhibitory stimuli.

Material and Method

Specimens of the dogfish, *Squalus acanthias*, 600 to 1500 grams in weight, taken during the month of August from Frenchman Bay, Maine, were used. The spinal cord was pithed posteriorly from the level of the sixth vertebra. The fish was secured ventral side up in a shallow tank of sea water. Although respiration continued in an apparently normal way, perfusion of the gills with sea water through the mouth was carried out in most cases, in order to insure an adequate supply of oxygen. The ventral aorta was exposed and ligated between its first and second branches. The former give rise to the first and second afferent branchial arteries which supply the hemibranch and the first holobranch. Therefore the second, third and fourth holobranchs were functional. Changes of pressure in the first branches and their derivatives were effected through a cannula inserted in the ventral aorta, anterior to the ligature, and connected with a burette

filled with a physiological solution (urea-saline, Lutz, 1930*b*). The heart beat was recorded on a smoked drum by means of a heart lever or a mercury manometer recording ventral aortic pressure through a cannula inserted posteriorly to the ligature.

Results

Cardiac inhibition was obtained when the first ventral aortic branches, and thus the gill vessels arising from them, were suddenly subjected to increases of internal pressure applied by opening the connection between the cannula and the burette for varying periods (Fig. 1, A). The amount of fluid entering during the application of pressure varied from 0.2 cc. to 5 cc., depending upon the height at which the burette was set and the length of time it was held open.

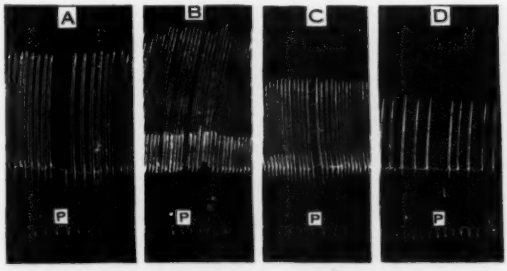

Fig. 1. Reflex cardiac inhibition following sudden increase of pressure within the gill vessels at *P*. Heart beat recorded by lever. In this and in subsequent figures the time record shows five-second intervals. Cord pithed posteriorly from the level of the sixth vertebra. Urea-saline solution in pressure burette except in B. A. Increase of pressure in the first branches of the ventral aorta. B. Same, with dogfish blood in the pressure burette. C. Same, with gill capillaries occluded with cornstarch so that no fluid entered. D. Increase of pressure in the third, fourth and fifth branchial arteries.

Inhibition was invariably obtained when the pressure applied (50 to 125 cm. of urea-saline) was obviously much higher than the dorsal aortic blood pressure. Simultaneous measurements of the ventral and dorsal aortic blood pressures showed that the pressure differences between the two aortae averaged 16.1 mm. Hg for systolic pressure and 4.3 mm. Hg for diastolic pressure, and that the pulse wave is transmitted through the gill capillaries to the dorsal aorta (Fig. 2, A). It is apparent, therefore, that the gill capillaries are relatively wide, and that the pressure in the afferent aortic system beyond the ligature is essentially the same as that in the dorsal aorta. Dorsal aortic systolic pressure ranged from 11 to 28 mm. Hg (8

measurements), and it was noted that fluid would not enter the ventral aorta from the burette below 25 cm. urea-saline (18.6 mm. Hg). A sudden increase of pressure averaging 10.7 mm. Hg above the average systolic pressure existing in the dorsal aorta was found to constitute an adequate stimulus for cardiac inhibition (Fig. 2, B), and in one instance a difference as low as 3 mm. Hg produced the response (Fig. 2, C).

Although urea-saline is considered to be a physiological solution, a control experiment was performed using heparinized fresh dogfish blood in the burette and its connections. A similar inhibitory response to increased pressure in the gill vessels was obtained (Fig. 1, B).

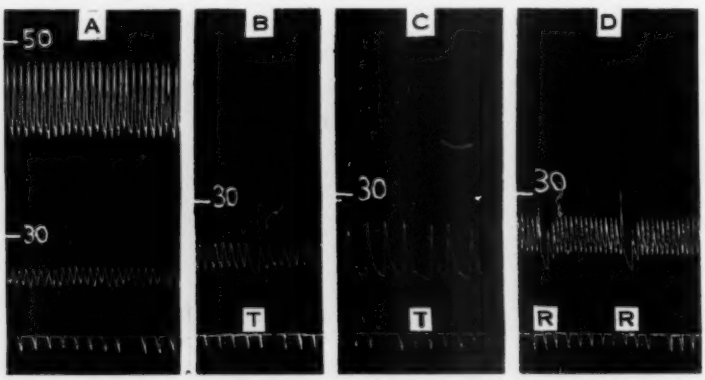

FIG. 2. Heart beat recorded by Hg manometer. Figures at left show pressure levels in mm. Hg. A. Simultaneous records of ventral (upper) and dorsal (lower) aortic blood pressure. B. Ventral aortic blood pressure record. Threshold increase of pressure in the first branches of the ventral aorta at *T* (33 cm. urea-saline, 24.6 mm. Hg). C. Same, threshold 25 cm. urea-saline (18.6 mm. Hg). D. Ventral aortic blood pressure record. Spontaneous ejection reflexes at *R*.

Cardiac inhibition was obtained when the burette was opened and immediately closed, allowing only 0.2 cc. of fluid to enter. Furthermore, holding the burette open, until 5 cc. of fluid had entered the ventral aorta, did not increase or continue the initial inhibition during the period of flow. It follows that the effective stimulus is the initial increase of pressure *per se*, and not flow of fluid through the gill vessels. This was substantiated by blocking the flow through the gill capillaries so that the application of pressure alone served to stimulate. Attempts to do this by tying the efferent branchial supply from the hemibranch and first holobranch were unsuccessful because of the numerous anastomoses between these vessels and the neighboring efferent system. An effective block on the afferent side was obtained

by occluding the gill capillaries with cornstarch, injected in suspension in urea-saline solution, through the ventral aorta. This suspension was withdrawn from the larger afferent vessels with a syringe and the vessels were refilled with urea-saline, thus giving a vascular pocket on the afferent side against which pressure could be applied without passage of fluid (Fig. 1, C).

That the receptors for the cardio-inhibitory response to increase of pressure within the gill vessels are not limited to the first two branchial arteries was shown by an experiment in which the ventral aorta was ligated between its first and second branches, and pressure applied to the third, fourth and fifth branchial arteries through a cannula inserted through the conus arteriosus (Fig. 1, D).

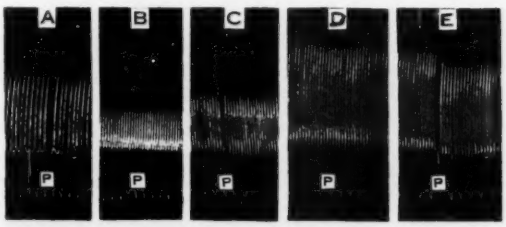

FIG. 3. Sudden increase of pressure within the first branches of the ventral aorta at *P*. Heart beat recorded by lever. A, before and B, after cutting the vagus supply to the heart on both sides. C. Vagus supply to heart cut on right side and increase of pressure in right gill vessels (crossed reflex). D, increase of pressure in right gill vessels, and E, in left gill vessels after cutting cranial nerves from fifth through tenth on right side (afferent supply from gills).

The cardiac inhibition following increase of pressure within the gill vessels is a reflex response inasmuch as it disappeared when the brain was destroyed by pithing. When the ramus posttrematicus of the fourth branchial division of the vagus (5th branchial nerve) and the ramus visceralis were cut on both sides the reflex was also abolished, thus showing that the efferent side of the reflex is in the vagus supply to the heart (Fig. 3, A, B). When these nerves were cut on the right side and the first right branch of the ventral aorta was clamped, thus leaving only the left gill vessels open to stimulation, the usual inhibition was obtained. When the clamp was shifted to the first left branch of the ventral aorta, the response was also obtained upon stimulating the right gill vessels (Fig. 3, C). Evidently the pathways for this reflex are both unilateral and crossed in the central nervous system. The threshold of pressure for the crossed reflex was about 10 cm. of urea-saline higher than that for the reflex obtained from stimulation of both branches.

The afferent innervation of the hemibranch and the first two gills was interrupted on both sides by cutting the first three branchial nerves (ninth and the first and second branchial divisions of the vagus). Following this no cardiac response to increase of pressure in both first branches of the ventral aorta was obtained. The integrity of the remaining cardio-inhibitory reflex mechanism was demonstrated by obtaining reflex inhibition upon pinching with forceps the skin between the fourth and fifth gill slits, the snout, the base of the pectoral fin, or upon cutting open the abdomen or scratching the wall of the pericardial chamber with a needle. The afferent side of the reflex following increase of pressure within the anterior gill vessels is thus located.

Inasmuch as cutting the first three branchial nerves involves opening the anterior cardinal sinus with considerable hemorrhage, the afferent nerve supply from the gills was interrupted on one side by transecting the cranial nerves from the fifth through the tenth close to the brain with a single incision through the skin. Following this, the usual cardio-inhibitory reflex was not obtained from the gill vessels on the operated side. Mechanical stimulation of the gill region on this side also failed to elicit a reflex, but both increase of internal vascular pressure and mechanical stimulation of the gills on the intact side evoked the cardio-inhibitory response (Fig. 3, D, E).

Discussion

The blood pressure in the elasmobranch has been found to be of the low pressure type. Thus Schoenlein (1895) found in *Torpedo* a branchial systolic pressure of 16 to 18 mm. Hg and in *Scyllium* one of 30 to 33 mm. Hg; Hyde (1908) found the mean pressure in a branch of the aorta of the skate to be 20 mm. Hg; Lyon (1926) found in sand sharks an average branchial pressure of 32 mm. Hg and an average dorsal or systemic pressure of 23.3 mm. Hg. In *Squalus acanthias* we found the average pressure in the ventral aorta to be 28.2 mm. Hg and that in the dorsal aorta to be 15.4 mm. Hg. The average pulse pressure in the ventral aorta, as recorded by the mercury manometer, was 13.3 mm. Hg which is 47.2 per cent of the average systolic pressure. The ventricular beat as a factor in maintaining blood pressure is obviously important. This is in accordance with the fact that no vasomotor innervation has been demonstrated in these fishes. The heart reflexes, therefore, must be of importance as regulatory factors for the vascular needs of the body. The cardio-inhibitory reflex, as shown above, can be elicited by an increase in gill blood pressure well within physiological limits, and may temporarily decrease the

diastolic blood pressure to a significant degree. In one case this decrease was 4 mm. Hg, which was 50 per cent of the diastolic pressure. Such a mechanism might come into play during an ejection reflex, when the sudden constriction of the branchial muscles forces water from the pharynx. The accompanying external pressure on the gill vessels would force blood from them in both directions. The blood pressure in the ventral aorta would rise, inasmuch as the blood would meet the valves in the conus arteriosus, evoking a sudden need for cardiac inhibition to prevent dangerous consequences, such as injury to the thin-walled afferent system. The increase in internal pressure would evoke the reflex, momentarily preventing additional pressure increase due to the ventricular action. As a matter of fact, our records show an increase of ventral aortic blood pressure well above threshold value followed by cardiac inhibition during spontaneous ejection reflexes (Fig. 2, D). This mechanism, therefore, being of physiological significance, may be compared with the carotid sinus mechanism in mammals. Cardiac inhibition during an ejection reflex may also be evoked by the mechanical stimulation of the gill region, inasmuch as the receptors for the heart reflexes have a wide distribution in the elasmobranch.

It is conceivable that in the course of evolution the widespread sensitive areas of the ancestral form, possibly typified by the elasmobranch, were concentrated or delimited until the condition seen in the mammal was reached. The carotid arteries of the mammal are derivatives of the primitive branchial system. The cardio-inhibitory reflex following increase of pressure within the gill vessels in elasmobranchs may exemplify, therefore, the evolutionary forerunner of the carotid sinus mechanism of mammals as it existed in whatever may have been the ancestral form. This is one of many instances in which it is apparent that physiological as well as morphological factors should be considered in evolutionary reasoning.

Summary

1. Cardiac inhibition follows sudden increase of pressure within the gill blood vessels of *Squalus acanthias*. An average increase of 10.7 mm. Hg above the average systolic pressure existing in the dorsal aorta constitutes an adequate stimulus for the inhibitory response.

Respiratory reflexes were frequently seen associated with the cardio-inhibitory reflex induced by changes in blood pressure. Inasmuch as the carotid-sinus mechanism in mammals has recently been shown to be concerned in the regulation of respiration, further work on the peripheral control of respiration through receptors within the gill vessels of elasmobranchs is in progress.

2. The cardio-inhibitory response is a reflex, with afferent pathways located in the branchial nerves and efferent pathways in the vagus supply to the heart. The reflex is both unilateral and crossed.

3. The average ventral aortic systolic blood pressure in *Squalus acanthias* is found to be 28.2 mm. Hg, the average dorsal aortic systolic pressure 15.4 mm. Hg, and the average ventral aortic pulse pressure 13.3 mm. Hg. The inhibitory reflex to increased ventral aortic pressure modifies the diastolic blood pressure to a significant degree.

4. The adaptive nature of the reflex is pointed out and its phylogenetic significance is discussed.

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THE EFFECT OF ADRENALIN ON THE BLOOD PRESSURE OF THE ELASMOBRANCH, SQUALUS ACANTHIAS

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In elasmobranch fishes the distribution and relative preponderance of the components of the autonomic nervous system and the effects of adrenalin on the heart and digestive tract differ considerably from the same factors in mammals, as pointed out by Lutz (1930*a*, 1930*b*, 1931). The effect of adrenalin (1 : 50,000 to 1 : 25,000) on the heart was found to be inhibitory and was interpreted as the response of an unbalanced parasympathetic mechanism in an organ lacking a sympathetic accelerator innervation (Lutz, 1930*a*). The well-developed chromaphil system seen in elasmobranchs (Lutz and Wyman, 1927) leads one to believe that it must be of functional significance, and a possible inhibitory emergency theory has been suggested to account for its existence (Lutz, 1930*a*). The lack of accelerator nerves to the heart and the failure to demonstrate a vasomotor innervation to the blood vessels makes the abundance of chromaphil tissue in these fishes of especial interest to the physiologist. The well-known effect of adrenalin on the blood pressure of mammals, an effect which is relatively brief and readily duplicated by successive doses, appears to differ from its effect on the blood pressure of cold-blooded animals. Bieter and Scott (1929) reported a rise of blood pressure in the frog following a dose of 0.2 cc. of epinephrine hydrochloride, 1 : 10,000, which persisted for at least one and one-quarter hours after the injection. MacKay (1931) has found that adrenalin causes a rise of ventral aortic blood pressure in skates (*Raia*), which is of much longer duration than the pressor effect in mammals. Herein are reported the results of a preliminary investigation of the effects of adrenalin on the vascular system of the elasmobranch, *Squalus acanthias*. In view of the system of branchial capillaries located between the ventral and dorsal aortæ, simultaneous records of the blood pressures in both systems were considered essential.

Material and Method

Specimens of the dogfish, *Squalus acanthias*, 730 to 1400 grams in weight, taken from Frenchman Bay, Maine, were used. The spinal cord was pithed posteriorly from the level of the sixth vertebra.

The fish was secured in a tank of sea water and the gills were perfused through the mouth as described in a previous paper (Lutz and Wyman, 1931). Simultaneous ventral and dorsal aortic blood pressures were recorded on a smoked drum by means of two mercury manometers and two cannulas, one inserted in one of the first branches of the ventral aorta thus leaving the second, third and fourth holobranchs on that side and all the gills on the other side for respiration, and the other inserted in the coeliac artery close to the dorsal aorta. The cannulas and manometer connections were filled with a physiological solution (urea-saline, Lutz, 1930*a*) containing a little heparin to prevent clotting. Adrenalin chloride (Parke, Davis and Co.), diluted in urea-

TABLE I

Effect of Initial Doses of Adrenalin

Exp. no.	Dose of adrenalin	Increase of pressure Ventral aorta			Increase of pressure Dorsal aorta		Change of heart rate
		Syst.	Diast.	Pulse	Syst.	Diast.	
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*beats per min.*
30	1 cc. 1 : 1000	71.5	40.0	150.0	75.0	—	−6
31	1 cc. 1 : 1000	29.1	30.8	27.8	53.8	72.8	+4
35	2 cc. 1 : 1000	70.0	46.1	114.1	91.0	80.0	0
36	2 cc. 1 : 1000	55.6	13.3	233.0	—	—	−30
27	2 cc. 1 : 10,000	39.2	38.5	40.0	111.8	133.3	0
29	2 cc. 1 : 10,000	60.0	33.3	100.0	66.5	—	−4
33	2 cc. 1 : 50,000	32.1	20.0	63.6	50.0	50.0	0
34	2 cc. 1 : 100,000	33.3	14.3	60.0	45.5	36.3	−4
37	2 cc. 1 : 500,000	33.3	42.8	23.1	91.6	111.0	+4

saline solution, was injected into the portal vein. MacKay (1931) has shown that in the skate the effects of adrenalin administered in this way are the same as those obtained by injection into a large systemic vein. Control injections of urea-saline solution and adequate controls for manipulation during injections were carried out.

RESULTS

Intravenous injections of adrenalin, in doses of one or two cc. of 1 : 1000 to 1 : 500,000, produced rises of ventral and dorsal aortic blood pressure, both systolic and diastolic, persisting for at least 30 minutes (Table I; Fig. 1, A). No attempt was made to determine the maximum duration of the pressor effect, but MacKay (1931) has found it to be from one to two and one-half hours in the skate. There was also a marked increase of pulse pressure. The effect on the heart rate was not constant, but there was a general tendency

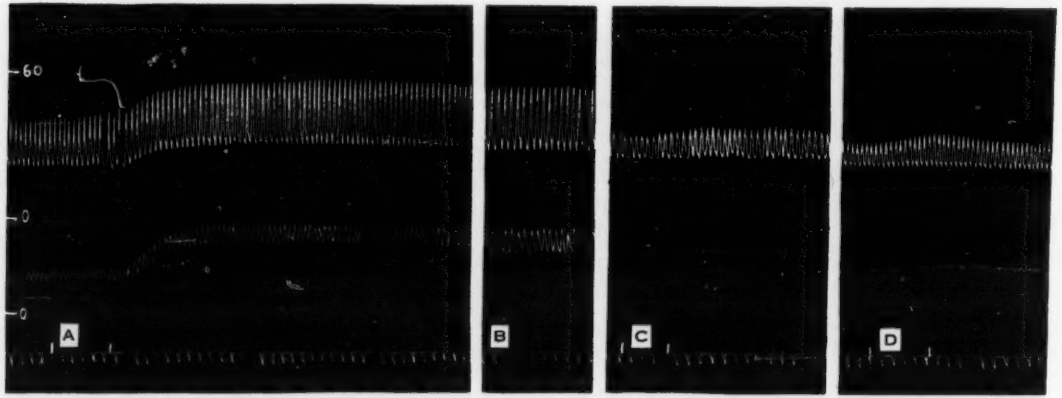

FIG. 1. The effect of adrenalin on the ventral (upper) and dorsal (lower) aortic blood pressures of *Squalus acanthias*. Cord pithed posteriorly from the level of the sixth vertebra. Time record shows five-second intervals. Figures at left show pressure levels in mm. Hg. The zero level (upper) for the ventral aorta is at 40 mm. Hg for the dorsal aorta. A. Exp. 27. Intravenous injection of 2 cc. of adrenalin, 1 : 10,000. B. Blood pressures 32 minutes later. C. Injection of 2 cc. of adrenalin, 1 : 10,000, 41 minutes after the initial injection (A). D. Exp. 33. Control injection of 2 cc. of urea-saline solution.

toward a decrease, especially following the stronger doses (1 : 1000, 1 : 10,000).

Subsequent doses of adrenalin following doses stronger than 1 : 500,000 produced small temporary increases of systolic pressure with little or no effect on the diastolic pressure, and consistent small increases of pulse pressure (Table II; Fig. 1, C). The rise of systolic

TABLE II

Effect of Subsequent Doses of Adrenalin
(For initial doses see Table I)

Exp. no.	Dose of adrenalin	Time after first dose	Increase of pressure Ventral aorta			Increase of pressure Dorsal aorta		Change of heart rate
			Syst.	Diast.	Pulse	Syst.	Diast.	
		min.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*beats per min.*
27	1 cc. 1 : 1000	33	1.8	11.1	4.3	17.1	16.7	−2
	2 cc. 1 : 10,000	41	2.7	0	8.3	3.8	0	0
	2 cc. 1 : 10,000	51	11.8	4.8	23.0	4.5	0	−2
29	2 cc. 1 : 10,000	7.5	2.6	0	5.9	4.4	—	−6
33	2 cc. 1 : 50,000	10	5.7	16.1	−5.9	15.0	23.5	0
34	2 cc. 1 : 100,000	3.5	3.1	0	6.2	6.2	6.7	+2
	2 cc. 1 : 100,000	8.5	3.1	0	6.2	6.7	0	−4
	2 cc. 1 : 1000	11.5	8.8	6.2	11.1	12.5	13.3	−4
37	2 cc. 1 : 500,000	9.5	20.0	11.1	33.3	33.3	12.5	0
	2 cc. 1 : 500,000	22.5	16.1	12.5	20.0	37.4	33.3	0
	2 cc. 1 : 1000	28.5	12.5	12.5	12.5	41.1	23.1	−6

pressure was, in most cases, actually due to the increase of pulse pressure. The effect of subsequent doses on the heart rate, however, was similar to that of initial doses.

Control injections of 2 cc. of urea-saline solution gave results which were essentially similar to those following subsequent doses of adrenalin with respect to blood pressure and pulse pressure. The heart rate, however, was unaltered (Table III; Fig. 1, D). It is probable, therefore, that such subsequent doses of adrenalin have little or no effect on the blood pressure.

After a dose of two cc. of adrenalin, 1 : 500,000, which gave a less prolonged pressor effect than stronger initial doses, subsequent doses of the same size were effective, producing significant increases of blood pressures and of pulse pressure which again were of shorter duration than the pressor effects of stronger doses (Table II, Exp. 37). This suggests that the ineffectiveness of adrenalin given during the prolonged pressor effect following stronger doses is due to the existence of the maximum pressor action of which the vessels are capable. Such

an explanation obviates the necessity of assuming that a pharmacological tolerance to adrenalin has been acquired.

Excluding cardiac effects, a change in ventral aortic pressure following the administration of adrenalin might be due to alterations in either the gill vessels or in the systemic vessels. The increase of blood pressure following initial doses of adrenalin was often associated with slowing or with no change in heart rate. The percentage increase of diastolic pressure in the dorsal aorta was consistently greater than that in the ventral aorta (Table I). These facts suggest a vasoconstrictor action of adrenalin, peripheral to the gill capillaries. Neither recording the venous outflow from the excised spiral valve (the blood vessels of which were perfused with adrenalin solutions) nor microscopic observation of the minute vessels of the tail, during

TABLE III

Effect of Control Injections of Urea-Saline Solution

Exp. no.	Dose	Increase of pressure Ventral aorta			Increase of pressure Dorsal aorta		Change of heart rate
		Syst.	Diast.	Pulse	Syst.	Diast.	
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*beats per min.*
31	2 cc.	−3.1	0	−5.2	0	0	0
33	2 cc.	16.7	5.0	40.0	17.6	12.5	0
33	2 cc.	9.6	5.0	18.2	10.5	5.5	0
34	2 cc.	4.2	−12.5	37.5	9.1	—	0
37	2 cc.	11.5	0	23.1	9.1	0	0
37	2 cc.	3.1	0	6.2	5.8	0	0

the injection or direct application of adrenalin, gave evidence of vasoconstriction. Further work is necessary to locate the region of action of adrenalin in producing the pressor effect in *Squalus acanthias*.

There is considerable doubt concerning the existence of a sympathetic vasoconstrictor innervation in elasmobranchs. The long sustained pressor action of small amounts of adrenalin and the accelerator and augmentor action of minute doses on the heart of the skate (Huntsman, 1931) suggest that a possible function of the well-developed chromaffin system is to take the place of sympathetic nervous factors in vascular regulation. However, the discovery by MacKay (1931) of pressor changes in the ventral aorta following sensory stimulation in spinal or anesthetized skates, which were too brief to be due to reflex discharge of adrenin, invites further search for vasoconstrictor nerves.

SUMMARY

1. Intravenous injection of adrenalin, in doses as low as 2 cc. of 1 : 500,000, produced long-sustained pressor effects in *Squalus acanthias*, together with marked increase of pulse pressure and a tendency toward decrease of heart rate.

2. Subsequent doses of adrenalin following doses stronger than 1 : 500,000 were ineffective. This is interpreted as being due to already existing maximum pressor action. Doses subsequent to a dose of 2 cc. of 1 : 500,000 were effective.

3. The pressor effect is interpreted as being due to extra-cardiac factors, peripheral to the gill capillaries, but the region of action of the adrenalin was not located.

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PROPERTIES OF THE BLOOD OF THE SKATE (RAIA OSCILLATA)

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The system developed in the skate for transport of oxygen and carbon dioxide is similar in some respects to that of man. Oxygen combines reversibly with hemoglobin and probably passes in and out of the blood by diffusion. Carbon dioxide, transported as bicarbonate, is prevented from greatly changing the blood reaction by the buffer function of proteins.

If one seeks a more detailed description of the skate's respiratory system, it is found to differ from that of man in many respects. Thus a labile body temperature introduces a degree of freedom in the skate's blood which is absent in the blood of normal man. Other differences depend upon the physical state of the environment. In the one case the blood is separated by a membrane from a moving liquid. From this liquid, oxygen in solution diffuses into the blood stream; into it, carbon dioxide passes directly. In the other case the lung, acting as a buffer between the blood and a variable external environment, maintains air nearly constant in temperature and composition in the ultimate areas where gas exchange takes place. Carbon dioxide passes from the blood, not into a virtual vacuum, as from the gills of the skate, but into a gas phase where the partial pressure of carbon dioxide fluctuates within narrow limits about a mean value of 40 mm. So much can be said by induction; it remains to be seen how well the properties of the skate's blood are adapted to the requirements.

The experimental methods used in this investigation have been described by Dill and Edwards (1931) and need not be discussed here. Two difficulties arose, both related to the character of the erythrocytes. These are very resistant to rupture and in determining oxygen content low values may be obtained because of incomplete hemolysis. When the quantity of saponin in the ferricyanide reagent was tripled, hemolysis was complete and oxygen could be determined satisfactorily. The other difficulty was a consequence of the high metabolic rate of these nucleated cells. After blood is equilibrated and sealed in sampling tubes a rapid decrease in its oxygen content occurs. Usually

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oxygen was determined immediately but occasionally delay was unavoidable. In order to correct for changes in oxygen content, the rate of oxygen consumption was determined at several temperatures.

Since these observations on metabolic rate of the blood may be of some interest in themselves they have been tabulated in Table I. In this experiment the blood had been saturated at a temperature of 10.5° with an oxygen partial pressure of 200 millimeters. Assuming the same solubility as in human blood of the same water content, dissolved oxygen amounted to 0.82 volume per cent and combined oxygen, 4.74 volumes per cent. With this information and the data of Table I and of Fig. 1 on the metabolic rates at 0, 20 and 40°, one can calculate the oxygen consumption of any specimen of blood

TABLE I

Oxygen Consumption by Skate's Blood

Initial oxygen content = 5.56 volumes per cent

Time min.	Oxygen Content vol. per cent		
	0° C.	20° C.	40° C.
21			4.70
96			1.88
115			1.64
140		3.70	
375		1.37	
1000	4.29		

within a wide range of conditions. It appears from Fig. 2 that the metabolic rate is not a linear function of the reciprocal of the absolute temperature. The observations are too few in number, however, to define this curve precisely.

Observations have been made on the oxygen dissociation curves of three specimens of blood (two of which were composited from several skates) at four temperatures. A preliminary experiment was carried out in the usual way with variable oxygen pressures and with carbon dioxide partial pressures ranging from 10 to 100 millimeters. Since the blood has about one-half the buffer value of human blood it was supposed that this range of carbon dioxide pressures would have a greater effect on the position of the oxygen curves than in human blood. On the contrary, the effect was too small to be evaluated. A second experiment (Blood B; temperature 10.4°; Table II) was carried out with a greater range in carbon dioxide pressures,—from 0.5 to 140 millimeters. With this extreme range there appeared to be a small decrease in affinity for oxygen with increasing acidity.

TABLE II

Equilibrium Values for Oxygen Absorption

	pCO_2	pO_2	Total O_2	HbO_2
	mm. Hg	*mm. Hg*	*vol. per cent*	*per cent of capacity*
Blood A. HbO_2 capacity 5.68 vol. per cent				
Temperature 37.5°	0.8	12.5	0.22	4
	1.0	48.2	1.54	25
	0.8	128	3.97	64
	1.3	248	5.29	82
Temperature 25°	0.8	14.6	0.41	6
	0.7	31.3	1.79	30
	0.7	84.6	4.53	75
	1.0	100	4.55	75
Temperature 0.2°	0.5	2.4	0.60	10
	0.6	10.8	2.80	48
	0.5	13.8	3.65	62
	0.7	24.3	4.94	85
	0.7	48.6	5.68	96
	1.5	150	6.43	100
Blood B. HbO_2 capacity 5.54 vol. per cent Temperature 10.4°	0.7	3.6	0.34	6
	0.7	5.3	0.32	6
	0.5	8.1	1.00	18
	1.5	16	2.20	39
	0.5	26.5	3.62	64
	0.6	40.1	4.56	79
	0.8	58.8	5.15	89
	1.1	76.8	5.54	95
	2.3	190	6.30	100
	10.7	25.8	2.68	47
	140	23.2	2.29	40
	141	33.0	3.07	53
	137	40.7	3.41	59
Blood C. HbO_2 capacity 4.18 vol. per cent Temperature 37.5°	2.2	108	2.60	56
	2.7	254	4.13	84
Temperature 25°	0.6	23.2	1.24	28
	0.5	48.9	2.50	56
	0.5	83.1	3.19	70
	0.6	108	4.04	89
	1.0	165	4.49	95
	1.6	212	4.75	98
Temperature 10.4°	0.9	18.8	2.49	58
	0.5	22.3	2.42	56
	0.6	38.9	3.14	71
	2.2	167	4.95	100

This effect is represented quantitatively in Fig. 3. Ordinarily it is convenient to express the relation between position of the oxygen dissociation curve and pH by such an equation as

$$\log (X_{50}) = a(\mathrm{pH}) + b,$$

where X_{50} represents the partial pressure of oxygen when the blood is 50 per cent saturated with oxygen, and the term a, the slope, is a measure of the rate of change of affinity of the blood for oxygen with rate of change in pH. This equation cannot be applied to

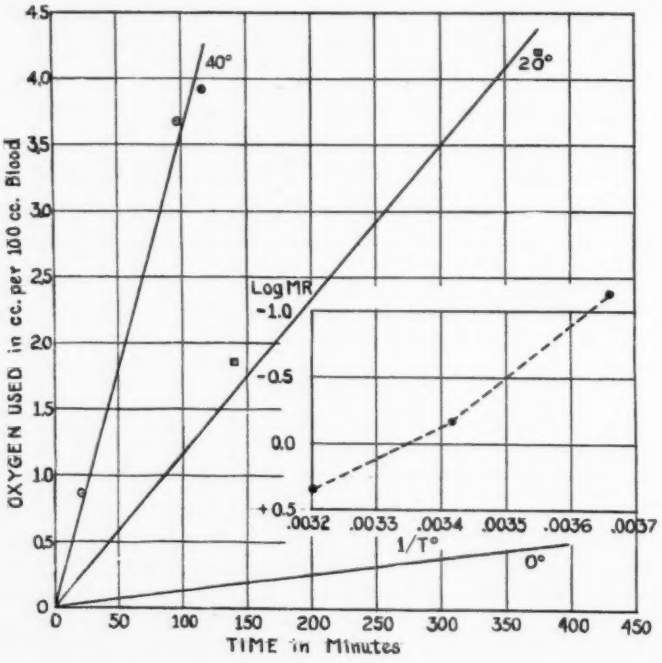

FIG. 1. Rate of oxygen consumption of normal skate's blood at temperatures of 0, 20, and 40°.

FIG. 2. (Inset.) Logarithm of the metabolic rate of skate's blood (oxygen used in cc. per hr. per 100 cc. blood) as a function of the reciprocal of the absolute temperature.

our data on skate's blood since we do not have direct determinations of pH nor knowledge of the value of pK′ for serum or for cells. For our purposes, however, it is enough to use calculated values for log $[(BHCO_3)_b/(H_2CO_3)_b]$. This quantity differs from pH by a

constant (or nearly constant) amount and hence the slope a will be essentially the same as though pH values were used. The curves for blood of the crocodile and of man shown in this figure are from Dill and Edwards (1931).

The curves for blood of the crocodile and for man are much steeper than for that of skate's blood. The contrast is greatest in the acid range; in fact within the range which corresponds to pCO_2 values from 10 to 140 mm. there is almost no change in position with change in reaction. However, the skate does not normally function within these limits but, as will be shown below, at a carbon dioxide pressure

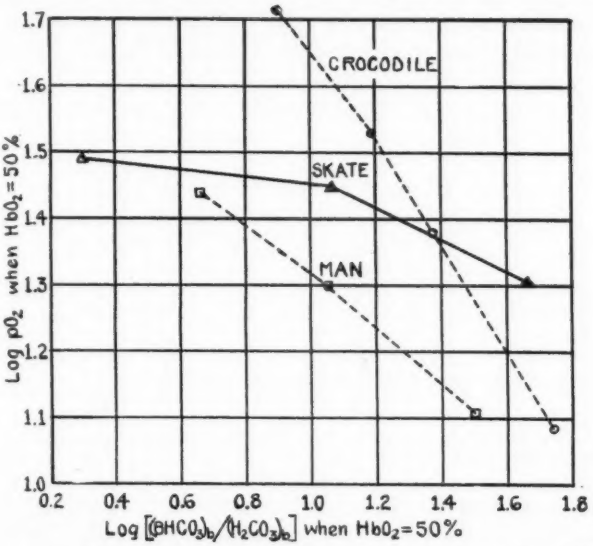

FIG. 3. Position of the oxygen dissociation curves (log pO_2 when $HbO_2 = 50$ per cent) in relation to log $(BHCO_3)/(H_2CO_3)$.

of one or two millimeters. In this range there is a distinct acid effect on oxygen affinity; here the slope a is roughly one-half as great as in man and one-fourth as great as in the crocodile. The blood of the skate is like that of *Urechis*, studied by Redfield and Florkin (1931), in the acid range but quite different when the reaction is more alkaline. They have found that the affinity of *Urechis* blood for oxygen is unaffected by change in hydrogen ion concentration when pCO_2 is varied from 0.5 to 92 millimeters. It would be interesting to speculate on the possible significance of these relationships in connection with structure of the hemoglobin molecule. However, we must remember

that the environment of hemoglobin is very different in these cases. It will be recalled that an abnormal value for a similar relationship was found in man in diabetic coma (Dill and others, 1929). Further discussion of this question had better wait, therefore, until it is possible to prepare these hemoglobins in the pure state and study them under strictly comparable conditions.

The data given in Table II have been used to construct the oxygen dissociation curves of Fig. 4. These have been drawn as members of the same family of curves, and aside from a few bad results the fit accords with this assumption. The effect of temperature on the affinity of blood for oxygen has been shown graphically for human blood by Brown and Hill (1923). Use has been made of their data and that of Fig. 4 in Fig. 5. This comparison indicates that q, the heat of reaction of 1 gram molecule of hemoglobin with n gram mole-

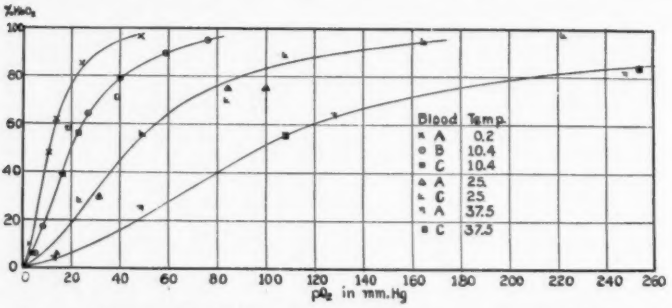

FIG. 4. Oxygen dissociation curves of skate's blood at temperatures of 0.2 10.4, 25 and 37.5° C. when $pCO_2 = 1 \pm 0.5$ mm.

cules of oxygen, is the same for the blood of the skate and of man. The significance of n remains somewhat obscure, but it is useful in characterizing the slope of the oxygen dissociation curve when $\log pO_2 = (f) \log Hb/HbO_2$. It has the value of 2.2 in man and in skate's blood it is slightly smaller, *viz.*, 2.0. If we accept the definition of n given by Brown and Hill, it follows that the value for Q, the heat evolved when 1 gram molecule of oxygen combines at constant volume with hemoglobin, is about the same as in human blood.

One must have a description of the carbonic acid dissociation curve of blood in order to understand the conditions under which carbon dioxide is excreted through the gills. It is known from the work of Collip (1920), confirmed by others (Kokubo, 1927 and Smith, 1929), that the carbonic acid content of selachian blood is no more than 10 to 12 volumes per cent. Accordingly the carbonic acid

dissociation curves have been studied over a low range of partial pressures. The curves for oxygenated blood are shown for temperatures of 10.4° (body temperature[2]) 25° and 37.5° in Fig. 6. The experimental procedure was simplified by the fact that, as in human blood, the relation of log (pCO_2) to log (Total CO_2) is linear or nearly so. Hence it was only necessary to determine a few points and fit the best straight line. The smoothed values were then transformed to the more familiar system of coördinates used in Fig. 6.

The alkaline reserve of blood, as suggested by Van Slyke and Cullen (1917), is most accurately defined by the bicarbonate content of arterial blood. When this is impractical, their method of equilibrating plasma of venous blood with alveolar air gives approximately correct values for human blood. The application of this method to fish blood, as by Collip (1920) and Kokubo (1927) does not define the alkaline reserve of fish blood but merely the carbon dioxide

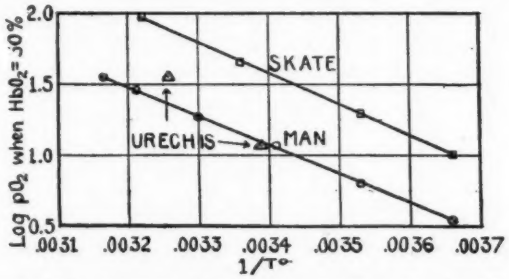

FIG. 5. Position of oxygen dissociation curves (log pO_2 when HbO_2 = 50 per cent) as a function of the reciprocal of the absolute temperature. The data for man are from Brown and Hill (1922–3) and for *Urechis*, from Redfield and Florkin (1931). In each case the oxygen is expressed in terms of partial pressures at the actual temperatures involved. The partial pressures of carbon dioxide were approximately 40 mm. for man, 1 for the skate and 12 for *Urechis*.

content at a partial pressure of carbon dioxide which is possibly twenty times greater than that of blood *in vivo*. It is probable that the values given by Collip for carbon dioxide content of blood equilibrated with atmospheric air measure the alkaline reserve more accurately than when alveolar air was used.

Having attained a description of oxygenated blood there remained to determine the effect of oxygenation on the position of these curves. There are several *a priori* reasons for supposing that this effect is too

[2] Body temperature was observed by rectum. It was maintained approximately constant by circulating sea water. Blood drawn at 10.4° and equilibrated at 25° and at 37.5° does not necessarily reflect the properties of blood drawn from animals acclimated to these higher temperatures. Possibly the blood would be altered in respect to available base and in other respects by change in body temperature.

small to be measurable in skate's blood. These are: (*a*) the hemoglobin is one-fourth its concentration in normal human blood and the effect of oxygenation on the carbonic acid dissociation curves of blood will be reduced accordingly; (*b*) the alkaline reserve is lower and the distance between the curves will on this account be low, as may be

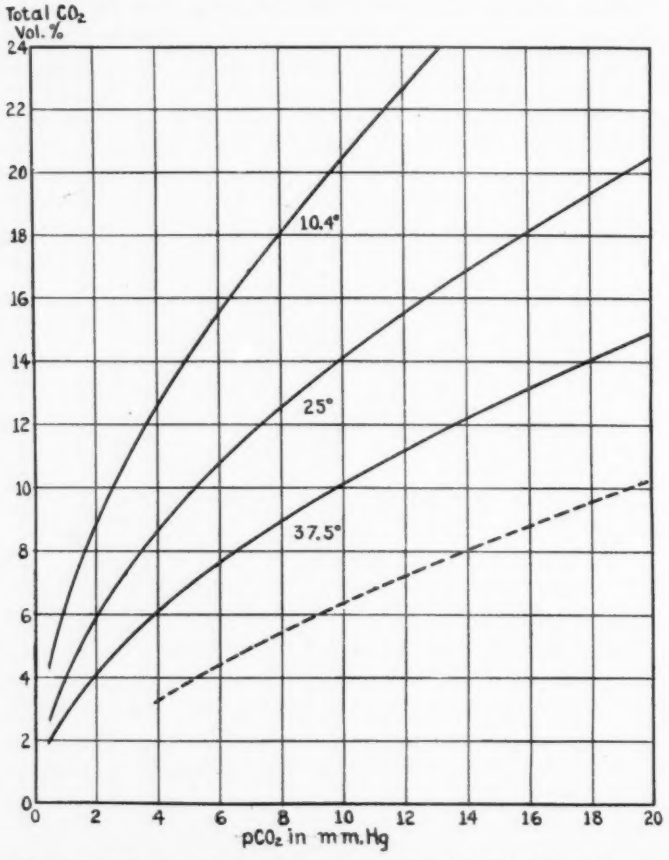

FIG. 6. Carbonic acid dissociation curves of skate's blood, body temperature 10.4°; equilibration temperatures, 10.4, 25 and 37.5°. The broken line corresponds to human blood at 37.5° in terminal chronic nephritis.

seen from the curves for human blood in diabetic coma (Dill and others, 1929); (*c*) the effect of acid on the oxygen dissociation curves is much less in skate's blood than in human blood (see Fig. 3); accordingly

the effect of oxygen on the base bound by hemoglobin should be correspondingly less. For all these reasons taken together it would appear that oxygenation should have little effect on the carbonic acid dissociation curves and in fact several experiments, including one on concentrated blood, revealed no significant difference between the curves of oxygenated and of reduced blood.

It appears, then, that in the skate carbon dioxide is transported principally by virtue of buffering properties of blood proteins and we shall now direct our attention to that subject. It will be convenient to consider first the buffer value of separated plasma. The results of experiments in which the carbonic acid dissociation curves were used to calculate buffer value of plasma specimens are shown graphically

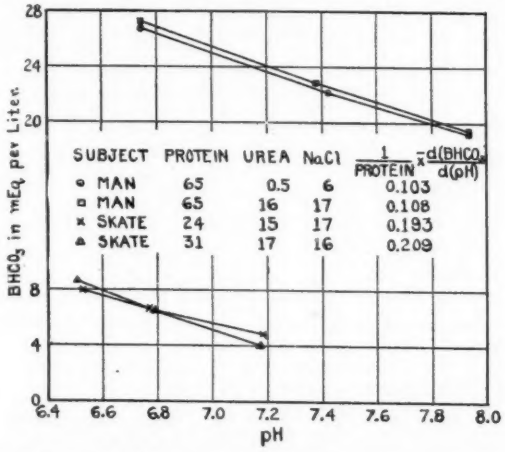

FIG. 7. Buffer value of skate's plasma and human plasma compared. Enough salt and urea was added to one specimen of human plasma to simulate skate's plasma in respect to those constituents. Concentrations of protein, urea and salt are in grams per liter.

in Fig. 7. Van Slyke's measure of buffer value is the rate of change of base bound with rate of change in pH:

$$\beta = \frac{dB}{d(\mathrm{pH})}.$$

These curves define the buffering capacity of plasma, but since this is due principally to protein, it has been divided by the protein concentration in each case. The calculations inserted in Fig. 7 indicate an unusually high value for the buffer value of plasma protein of the skate. To determine the extent to which this is due to the high

concentrations of urea and of sodium chloride in skate's plasma, a human plasma was prepared with concentrations of these substances typical of the skate. The effect of this modification proved to be very small and it follows that the proteins of the skate's plasma have, per gram, about twice the intrinsic buffering value of human plasma proteins. It will be noted that the protein concentration in the skate's plasma is from one-third to one-half as great as in human plasma. In effect, then, the buffer value of plasma per unit volume is almost equal in the two species.

It is possible to compare the buffer value of skate's whole blood with that of human blood by reference to the chart of Henderson and associates (1930, Fig. 3). The observations given in Table III are

TABLE III

Buffer Value of Oxygenated Skate's Blood at 37.5°

Blood	HbO_2 Capacity	CO_2 Capacity at $pCO_2 = 40$ mm.	$\Delta CO_{2_{8-20}}$		
			Observed	Calculated from human blood *	Ratio
	mEq. per l.	*mEq. per l.*	*mEq. per l.*	*mEq. per l.*	
Normal	2.0	8.4	3.3	3.1	1.1
Concentrated	4.7	7.0	3.15	3.2	1.0

* These values are calculated from the empirical chart of Henderson and associates (1930, Fig. 3).

based on one specimen of normal blood from the skate and one concentrated specimen obtained by centrifuging normal blood and removing about one-half the plasma. The comparison indicates that skate's blood has about the same buffer value as human blood of the same oxygen-combining capacity. Since the ratio of cell proteins to oxygen-combining capacity is about one-half greater in the skate than in man, it appears that the buffer value of cell proteins per unit weight is much less in the skate than in man.

This information regarding the physicochemical properties of skate's blood constitutes a suitable basis for study of changes in the respiratory cycle. The additional observations required are the oxygen and carbon dioxide contents of arterial and venous blood. We succeeded in obtaining for this purpose blood from the dorsal aorta and from the conus arteriosus while sea water at body temperature (9 to 10° C.) was being circulated over the gills. By application of the data thus obtained to the carbonic acid and oxygen dissociation

curves it is possible to calculate the partial pressure of carbon dioxide and of oxygen in the blood *in vivo*. This and other calculations are shown in Table IV where a comparison also is made between respiratory changes in the skate and in man in terminal chronic nephritis (Henderson and others, 1927), a state which approximates in many respects to that of the normal skate.

The data given in Table IV have several points of interest. The

TABLE IV

Comparison of the Skate with Man in Terminal Nephritis

	Skate *	Man †
Body temperature, ° *C.*	10.4	37.5
Cell count, *million per mm.*³	0.2	1.0
Red cell volume, *cc. per 100 cc.*	20.0	14.7
Oxygen capacity, *vol. per cent*	6.00	5.60
Free oxygen, arterial blood, *vol. per cent*	0.32	0.28
Combined oxygen, arterial blood, *vol. per cent*	5.58	5.32
Combined oxygen, arterial blood, *per cent of capacity*	93	95
Free oxygen, venous blood, *vol. per cent*	0.07	0.06
Combined oxygen, venous blood, *vol. per cent*	1.91	1.62
Combined oxygen, venous blood, *per cent of capacity*	32	29
CO_2 content, arterial blood, *vol. per cent*	7.70	8.61
CO_2 content, venous blood, *vol. per cent*	10.84	11.75
CO_2 transport, *vol. per cent*	3.14	3.14
O_2 transport, *vol. per cent*	3.92	3.92
pCO_2, arterial blood, *mm. Hg*	1.3	15
pCO_2, venous blood, *mm. Hg*	2.6	23
pO_2, arterial blood, *mm. Hg*	70	110
pO_2, venous blood, *mm. Hg*	14	27
pH_s, arterial	7.82 ‡	7.00
pH_s, venous	7.67 ‡	6.95
ΔpH_s	0.15	0.05

* While these data given for the skate are based on a specimen of arterial blood from one skate and of venous blood from another, observations on other individuals have verified the approximate accuracy of the figures given in the table.

† These values have been obtained directly or by calculation from the study of terminal nephritis by Henderson and associates (1928). The values for carbon dioxide and oxygen transport have been arbitrarily made equal to the observed values in the skate. Associated changes in dependent variables have been read from the alignment chart for blood in nephritis.

‡ These values for pH_s are calculated on the assumption that $pK'_s = 6.24$ under the experimental conditions. The absolute values for pH_s may be incorrect, but the value for ΔpH_s will not be affected.

striking difference as compared with man is in the carbon dioxide partial pressure. Man in terminal nephritis has a greatly increased rate of pulmonary ventilation but the partial pressure of carbon dioxide cannot be kept below 15 mm.; the skate, on the contrary, keeps the carbon dioxide pressure below 2 mm. in arterial blood. The steep character of the carbonic acid dissociation curve in this

range makes possible the transport of 3 volumes per cent of carbon dioxide with a change in its partial pressure of only 1.3 millimeters. Another point of interest is the large change in pH of skate's blood. This is no doubt related to the greater effect in human blood of oxygenation on base bound by hemoglobin.

These facts are of particular interest in connection with the equilibrium between blood and air in respect to oxygen and carbon dioxide. The oxygen dissociation curve of skate's blood at 10.4° is approximately the same as man's at 37.5° and the oxygen tension in sea water is approximately the same as in air. The fact that arterial blood of the skate has about the same percentage saturation with oxygen as that of man indicates that the adequacy of oxygen transfer is approximately the same in the two species.

It has been shown by Bock and Field (1924) that in man the carbonic acid pressure is about the same in alveolar air as in arterial blood, most of the differences in partial pressure being within ± 1 millimeter. It is now possible to say that the partial pressure of carbon dioxide in the arterial blood of the skate exceeds that in water passing over the gills by no more than 1 or 2 millimeters. Collip has suggested that it is possible that a steep pressure gradient exists "*between the dissolved carbon dioxide in the blood on the one side, and in the sea water on the other.*" Such may be true of some bony fishes but it is not true of the skate.[3] His argument is as follows:

"As the hydrogen ion concentration of sea water is in most instances lower than that obtaining in the blood of marine forms and as the bicarbonate content of the latter is much higher than that of the former it is evident that the amount of the dissolved carbon dioxide in the blood or body fluids of marine forms must be considerably greater than that occurring in sea water. The tension of carbon dioxide in the blood of marine forms must also be proportionately higher than that in sea water."

This argument is sound provided one assumes that sea water is in equilibrium with atmospheric air in respect to free carbon dioxide. It may be misleading, however, because of the use of the word "considerably." Let us make a specific comparison of skate's blood and sea water:

	$BHCO_3$, *vol. per cent*	pCO_2, *mm. Hg*
Sea water	5	0.2
Arterial blood of the skate	8	1.4

[3] In advance of its publication, we have had the privilege of reading the paper by Root (*Biol. Bull.*, **61**: p. 427), on the respiratory function of the blood of marine fishes. His single observation on arterial blood of *Prionotus carolinus* shows substantially the same pressure gradient of carbon dioxide from blood in the gills to sea water as we have found in the skate.

Assuming the same pK′, the blood of the skate will be more acid by 0.64 pH units. It is true that the ratio of carbon dioxide pressures is 7 to 1 but pressure gradient depends not on the ratio but on the difference in pressure. This difference, 1.2 mm., is small,—of the same order as in man.

Summary

In the acid range, carbon dioxide pressure has almost no effect on affinity of skate's whole blood for oxygen. In the physiological range the effect is appreciable but still only one-half as great as in man. No difference was discerned between the carbon dioxide dissociation curves of oxygenated and of reduced blood. This was partly due to the facts that the hemoglobin concentration is one-fourth as great as in man and that the carbonic acid-combining capacity (when $pCO_2 = 40$ mm.) is less than in man.

The effect of temperature on the oxygen dissociation curves is identical with that found by Brown and Hill (1923) for human blood but somewhat different from that found by Redfield and Florkin (1931) for *Urechis* blood.

The buffer value of plasma proteins is about twice as great, per unit weight, as that of human plasma proteins. Since the concentration of protein in skate's plasma is one-third to one-half as great as in human plasma, it follows that the buffer value of plasma of the two species is about the same. Buffer value of whole blood is nearly equal to that of human blood of the same oxygen-combining capacity.

Transfer of gases between the blood and the external medium takes place under conditions which are quite different from those in the lungs of man. Nevertheless arterial blood is about equally saturated with oxygen in the two species. The absolute values for carbon dioxide pressure in man and the skate are very different because the blood of the skate is exposed to a virtual vacuum in respect to carbon dioxide. The pressure head of carbon dioxide from blood to the external medium, however, is of the same order of magnitude, about 1 mm. in each species. The supposition that there is a steep pressure gradient in respect to carbon dioxide in such a marine species as the skate is incorrect.

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THE NATURE OF THE REDUCING SUBSTANCES IN THE BLOOD SERUM OF LIMULUS POLYPHEMUS AND IN THE SERUM, CEREBROSPINAL FLUID AND AQUEOUS HUMOR OF CERTAIN ELASMOBRANCHS

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From the data in the literature it seems to be generally agreed that a variable part of the material determined as blood sugar is not glucose. Different investigators have attempted to determine glucose alone by using different reagents that react only with glucose, or by removing interfering substances (West, Scharles and Peterson, 1929) (Somogyi and Kramer, 1928). Folin and Svedberg (1926), because of discrepancies in the residual reducing substances after fermentation as determined by the Folin and Wu (1920) and Folin (1926) methods, say that the fermentable sugar is not all glucose. Both Benedict (1928) and Somogyi and Kramer (1928) conclude that there is no measurable amount of fermentable sugar other than glucose present in human blood.

We are presenting in this paper evidence to show that the reducing substances in the blood of *Limulus polyphemus* and in the blood, cerebrospinal fluid and aqueous humor of certain elasmobranchs are fermentable by yeast, and not appreciably affected by hydrolysis. We have determined the total reducing substances, reported as mg. glucose per 100 cc., by the method of Folin and Wu (1920) in the serum of *Limulus polyphemus* in twelve instances; in the serum of elasmobranchs in seven instances, in their cerebrospinal fluid in five, and aqueous humor in two instances. Yeast fermentation was done by the method described by Benedict (1928) in all these cases. The yeast blank which was subtracted from the residual after fermentation ranged from 4 to 6 mg. per 100 cc. Hydrolysis was done according to the method of Folin and Berglund (1922) in the twelve specimens of *Limulus polyphemus*.

We have already reported data on the relative composition of sea water and the blood of *Limulus polyphemus* (Dailey, Fremont-Smith and Carroll, 1931). The data reported here were obtained from the same samples of blood. The blood was taken by inserting a needle

into the body cavity at the attachment of the caudal spine. In the elasmobranchs we endeavored to obtain samples allowing as little asphyxia as possible. The aqueous humor was obtained by puncture of the anterior chamber of the eye. Cerebrospinal fluid was obtained by puncture through the anterior fontanelle while the gills were still immersed in running sea water.[1] To collect blood the fish was then removed from water, the abdominal wall rapidly slit, the viscera pushed aside, and blood aspirated from the inferior vena cava. In animal 7, Table II, blood was obtained also from the abdominal aorta. The blood was collected under oil, centrifuged, and a "protein-free filtrate" made at once.

TABLE I

Reducing Substances in the Serum of Limulus Polyphemus

Animal	Date	Total Reducing Substances *mg. glucose per 100 cc.*	Residual after Fermentation *mg. glucose per 100 cc.*	After Hydrolysis *mg. glucose per 100 cc.*
1	8/12/29	10	0	10
2	8/12/29	11	3	13
3	8/13/29	15	2	15
4	8/13/29	11	3	15
5	8/13/29	12	1	11
6	8/13/29	14	3	18
7	8/26/29	14	0	14
8	8/26/29	21	1	21
9	8/26/29	16	0	21
10	8/29/29	13	2	13
11	8/29/29	22	0	22
12	8/29/29	15	1	19

The data on *Limulus polyphemus* are presented in Table I; those on the elasmobranchs in Table II. In the sera of *Limulus polyphemus* the residual reducing substances after fermentation varied from 0 to 3 mg. per 100 cc. and averaged 1.5. In the fluids from elasmobranchs there was in one instance a residual of 5 mg. per 100 cc., but the average was only 2 mg. per 100 cubic centimeters. The greatest change in reducing substances of the blood of *Limulus polyphemus* with hydrolysis was 5 mg. glucose per 100 cc., while in eight of the twelve cases there was a change of 2 mg. per 100 cc. or less. These changes are too slight to be of significance. Considering the limits of error of the methods used it may be said that the total reducing substances in serum, cerebrospinal fluid or aqueous humor examined

[1] There is some question as to whether this fluid, obtained from the perimeningeal spaces, is truly analogous to the mammalian cerebrospinal fluid. (Smith, H. W., 1929. *Jour. Biol. Chem.*, **81**: 407.)

TABLE II

Reducing Substances in the Serum, Cerebrospinal Fluid and Aqueous Humor of Elasmobranchs

Animal	Date	SERUM		CEREBROSPINAL FLUID		AQUEOUS HUMOR	
		Total Reducing Substances *mg. glucose per 100 cc.*	Residual After Fermentation *mg. glucose per 100 cc.*	Total Reducing Substances *mg. glucose per 100 cc.*	Residual After Fermentation *mg. glucose per 100 cc.*	Total Reducing Substances *mg. glucose per 100 cc.*	Residual After Fermentation *mg. glucose per 100 cc.*
1. Mustelis canis	8/5/29	190	2				
2. Mustelis canis	8/7/29	157	1	107	1	50	0
3. Raia ocellata	7/18/29			23	0		
4. Carcharias littoralis	8/12/29	70	4	52	4		
5. Carcharias littoralis	7/29/29	70	1				
6. Carcharias littoralis	8/2/29	71	2	64			
7. Carcharinus obscurus	7/29/29	36	2	60	4		
		(aorta 73)	(aorta 5)				
8. Carcharinus obscurus	7/29/29	138	1	93	0	35	1

are fermentable by yeast and not changed by mild hydrochloric acid hydrolysis.

The striking difference in the level of total reducing substances in the blood of *Limulus polyphemus* and of the elasmobranchs studied is interesting in relation to the relative activity of the two groups. This relationship was discussed by Gray and Hall (1930). From their investigation we would expect to find low blood sugars in the sluggish and inactive *Limulus* and higher sugar values in the more active elasmobranchs. Our findings are consistent with theirs, as seen in Tables I and II. It is well-known that asphyxiation produces an increase in the amount of sugar in the blood of fishes (Denis, 1922; Menten, 1927; and Scott, 1921), which may in our cases have accentuated the higher blood sugar level of the elasmobranchs.

The fact that a fermentable reducing substance, not affected by hydrolysis, and therefore probably glucose, is consistently found in the blood of *Limulus polyphemus* and these elasmobranchs is interesting because there is little information as to the concentration or nature of the reducing substances in the blood of lower animals, and because of the ancient lineage of both species. We found the level of glucose in the sera of *Limulus polyphemus* to be relatively constant, varying only from 10 to 22 mg. per 100 cc. in the twelve animals studied.

There are some data in the literature on the blood sugar of elasmobranchs. Denis (1922) found the sugar of *Mustelis canis* to vary from 80 to 181 mg. per 100 cc.; Gray and Hall (1930) found 65 and 87 mg. per 100 cc. in two dogfish. These figures are within the limits of our findings, but we do not know the relationship of the blood sugar level, which varied from 36 to 190 mg. per 100 cc., to the degree of asphyxiation in the animals studied.

In Table II are reported five instances of parallel determinations of reducing substances in serum and cerebrospinal fluid. In four of these cases total and fermentable reducing substances were distinctly higher in venous serum than in cerebrospinal fluid. In case 7 the venous blood was lower, but the arterial higher. These studies give us no indication as to the relationship between reducing substances in the serum and cerebrospinal fluid at equilibrium as asphyxial elevations of the blood sugar had undoubtedly occurred after the cerebrospinal fluid was removed.

Conclusion

The reducing substance found in the blood serum of *Limulus polyphemus*, and in the blood serum, cerebrospinal fluid and aqueous humor of elasmobranchs is fermentable by yeast and not appreciably

affected by hydrochloric acid hydrolysis. It is, therefore, probably glucose.

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STUDIES ON CELL METABOLISM

I. The Oxygen Consumption of Nereis Eggs Before and After Fertilization

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The sudden and enormous increase in the oxygen consumption of *Arbacia* eggs soon after fertilization, which was observed by Warburg (1908) and Shearer (1922*a*) and confirmed by measurements of the heat production (Shearer, 1922*b*, Rogers and Cole, 1925), had led to the assumption that the fertilization phenomena require a great expenditure of energy. Shearer correlates this increase to cortical changes in the egg and to the formation of the fertilization membrane. Loeb attributed the increase to the fact that the unfertilized eggs of the sea urchin are usually in the resting stage. To prove the validity of his hypothesis, he measured in collaboration with Wasteneys (1912) the oxygen consumption of fertilized and unfertilized starfish eggs, which as a rule are immature when taken out of the ovary, but as soon as they are placed in sea water may become mature. No noticeable increase in the rate of oxidation of fertilized starfish eggs was observed by these investigators, since they postulated "those oxidations which lead to nuclear division were already going on in the eggs at the time the spermatozoa entered." As Lillie and Just (1924) point out, with the formation of the hyaline plasma layer, the egg of *Arbacia* may be said to be well started on its first cleavage cycle. This would mean that "the measured oxygen consumption belongs rather to the physiology of cell division than to the more specific events of fertilization." (Lillie and Just, 1924.)

It was therefore of particular interest to the physiology of fertilization, to measure the respiratory changes of eggs in which this process can be observed from its initial stages. The eggs of the annelid *Nereis* are particularly suited to this purpose. As the excellent studies of Wilson (1892) and F. R. Lillie (1912) have established, the ovum of *Nereis* eggs is inhibited at the end of its period of growth. The germinal vesicle undergoes none of the preparatory stages of maturation unless the egg be fertilized. The spermatozoön remains more or less quiescent within the egg during the completion of the

maturation division and the internal events of fertilization are resumed after the formation of the second polar body.

Experimental

The experiments were performed in Woods Hole during the summers of 1929 and 1930. Those of 1929 were done with the collaboration of Dr. A. Tyler. All the experiments were performed with freshly-caught *Nereis*. Large flat-bottomed Warburg vessels with Barcroft micro-manometers were used for the measurement of the oxygen consumption. The temperature of the water bath was kept at 25° C. The eggs were placed in the main side of the vessels. In some cases the sperm were placed in the side arm and fertilization was performed by pouring the sperm into the main side of the vessel, after the oxygen consumption of the unfertilized eggs had been

Table I

Oxygen consumption of Nereis eggs, before and after fertilization. The oxygen consumption after fertilization was followed until the two-cell stage had been reached.

Oxygen consumption in cu. mm. per hour		Per cent division
Before fertilization	After fertilization	
44.5	45.2	70
37.0	35.1	65
51.3	48.2	69
53.1	52.4	60
48.1	49.2	63
10.8	9.1	68
6.8	6.5	70
14.6	15.0	70
13.5	13.9	69

measured. In other experiments, the sperm were added directly by opening the vessel. The results obtained by both methods were identical. A total of forty-two experiments were performed.

The oxygen consumption after fertilization was measured until the eggs had reached the two-cell stage, *i.e.*, for about 80 minutes. The maximum percentage of cell division observed was 70 per cent and the minimum 45 per cent. In Table I are given figures of some of these experiments, at hourly periods. Figure 1 shows the oxygen consumption at different intervals after fertilization. In most cases (about 80 per cent) there was an average increase of 25 per cent in the oxygen consumption during the first eighteen minutes after fertilization. According to Lillie's observations on the fertilization events of *Nereis*,

this corresponds to the time when the fertilization cone has flattened out, but the spermatazoön is still external to the membrane. But if one compares the total oxygen consumption of fertilized eggs (from the moment fertilization takes place, until the two-cell stage has been reached) with the oxygen consumed by the same eggs when unfertilized in equal length of time, it is observed that there is no difference. The subsequent decrease in the oxygen consumption is possibly due to a diminution in the rate of diffusion of the CO_2 produced by the cells on account of the jell which surrounds them. The amount of jell secreted by the ovum increases from the time of fertilization.

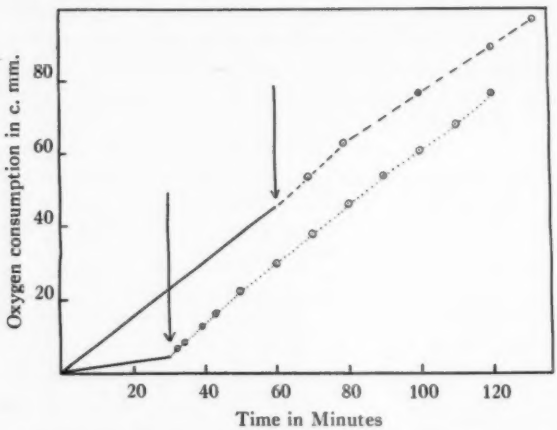

FIG. 1. The oxygen consumption of *Nereis* eggs before and after fertilization, as compared to the oxygen consumption of sea urchin eggs before and after fertilization. *Arbacia*, the lower line. *Nereis*, the upper line. Arrows mark the time of fertilization.

DISCUSSION

The striking increase in the oxygen consumption observed soon after *Arbacia* eggs have been fertilized has exercised such influence in the minds of most biologists, that one always finds it stated that the fertilization phenomenon is *in general* accompanied by an enormous increase in the oxidative processes of the fertilized cell. The case of fertilized starfish eggs, where such an increase is not observed, has not received corresponding attention. If Loeb's explanation for this lack of increased oxidation in fertilized starfish eggs is correct, one would expect in fertilized *Nereis* eggs about the same increase in the oxygen consumption as found in fertilized sea urchin eggs, since the initial process of fertilization starts here only when the spermatozöon enters the ovum. As has been shown in the experimental part, such is not

the case. All one can say is that during the first eighteen minutes (which corresponds to the formation of the fertilization cone in the egg protoplasm while the spermatozoön is still found external to the substance of the entrance cone) there is a small increase of about 25 per cent, which is negligible compared to that found in fertilized sea urchin eggs where the oxygen consumption goes up to eight times that before fertilization. Fertilization in *Nereis* eggs proceeds without a decided increase in the cellular oxidative processes just as in starfish eggs. If expenditure of extra energy is needed for the performance of this activity, it is possibly supplied through a hydrolytic process, probably glycolysis. In the breakdown of glycogen to lactic acid an amount of energy is liberated, which according to Burk's calculations (1929) is from one and a half to two times greater than the heat of reaction. The great increase in the oxygen consumption of *Nereis* eggs after the addition of a reversible dye seems to support the view that these eggs possess a carbohydrate fermentation process, since the catalytic effect of dyes is mainly due to oxidation of carbohydrates which have already been rendered easily oxidizable by the action of enzymes (Barron, 1929).[1]

Conclusion

The oxygen consumption of *Nereis* eggs before and after fertilization to the two-cell stage has been measured.

1. The total oxygen consumption, from the initiation of fertilization to the two-cell stage, is no greater than the oxygen consumption for a similar period of time before fertilization.

2. When the oxygen consumption is measured at shorter intervals there is a small increase of about 25 per cent during the first eighteen minutes.

3. The present observations, together with those on the starfish eggs reported by Loeb, suggest that the enormous increase in oxygen consumption of fertilized sea urchin eggs may be considered an exception.

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[1] These experiments were performed with cresyl blue as catalyst. The increase in the oxygen consumption after dye addition is about 300 per cent.

THE EFFECT OF ANAËROBIOSIS ON THE EGGS AND SPERM OF SEA URCHIN, STARFISH AND NEREIS AND FERTILIZATION UNDER ANAËROBIC CONDITIONS

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The effect of anaërobiosis upon fertilization has been studied by various authors using the sea urchin as material for experimentation. All these studied have been confined to the effect of lack of oxygen after the sperm and the egg had been in contact, missing therefore the initial process of fertilization. Loeb and Lewis (1902) exposed the eggs of *Arbacia* soon after fertilization to lack of oxygen and stated that the process was inhibited. Mrs. Harvey (1927) subjected the eggs of the same species to a stream of hydrogen in a "modified Engleman's chamber" at different times after fertilization. In these experiments anaërobiosis was not reached according to the author until twenty minutes later. It is also possible that the sea water became hypertonic because of the small amount of fluid used in the chamber. More recently, Mrs. Harvey studied the effect of lack of oxygen on the eggs and sperm of sea urchin and on fertilization (Harvey, 1930).

In order to study the effect of anaërobiosis upon the fertilization phenomena, it is essential in the first place to select eggs and sperm of species which can resist the lack of oxygen without injury, at least during the time before they are brought in contact; in the second place, to use a technique where the absence of oxygen is controlled and where there is no danger of the sea water becoming hypertonic. It is the purpose of this paper to describe such a technique and to relate the effects of anaërobiosis upon the initial phase of fertilization of the following species: sea urchins, starfish and *Nereis*. These experiments were performed in Woods Hole during the summer of 1929.

Method for Studying the Effect of Anaërobiosis on Fertilization

Three large specimen bottles (150 cc. capacity), tightly stoppered with rubber stoppers and connected to each other through glass tubing,

were used for every experiment. Bottle A contained water in order to saturate the gas. The gas inlet was on bottle A. Bottle B contained the eggs suspended in 50 cc. of sea water. These eggs were previously received in large crystallizing dishes, washed twice with sea water and passed through cheesecloth. Bottle B contained a spoon where the sperm was kept when the fertilization was performed with sperm in anaërobiosis or a burette containing boiled sea water, when the fertilization was performed with fresh sperm. In the latter case there also was a platinum electrode and a saturated KCl bridge connected to a saturated calomel electrode. Bottle C contained a solution of safranin (approximate E′o − 0.290 v.) in Sorensen's phosphate buffer pH 8.0 and some platinum asbestos.

Test for Absence of Oxygen

It is essential in an experiment where the effect of anaërobiosis is followed, to be reasonably sure that no detectable amounts of oxygen are present in the vessels where the experiments are performed. In the early days reduced methylene blue had been used for such a purpose, but this is an unreliable test. The absence of luminescence in *Cypridina luciferin-luceferase* or in luminous bacteria has been advocated by Harvey (1926). When reversible systems showing a change of colour or light from the oxidized to the reduced state are used as tests for the absence of oxygen, it is necessary to have in mind that the rate of oxidation of the system is about proportional to the E′o of the system. Reduced indophenol will remain reduced for some minutes when the vessel containing the leuco dye is exposed to the air. Reduced methylene blue will oxidize more quickly but will remain reduced for a time in the presence of small oxygen concentrations. The more negative the E′o of the dye is, the more easily it will be oxidized by oxygen when in the reduced state (Barron, 1931). On these grounds the writer has used and recommends reduced safranin T as an excellent test for detecting the presence of minute traces of oxygen.

When fertilization was performed with fresh sperm the E. M. F. of the sea water obtained through a bright platinum electrode against a saturated calomel electrode was also used to detect the presence of oxygen. It is known that this electrode is extremely sensitive to oxygen.

Safranin was reduced with hydrogen and platinized asbestos. After complete reduction had been obtained, nitrogen (purified according to Michaelis and Flexner, 1928) was bubbled throughout the experiment. Experiments in which there was the slightest coloration of safranin were discarded.

The Effect of Anaërobiosis on the Eggs and Sperm of Sea Urchin and Fertilization under Anaërobic Conditions

Loeb (1915) stated that sea urchin eggs could be better preserved in absence of oxygen, and related experiments where eggs kept for twenty-four hours under hydrogen could be fertilized as well as fresh eggs. It is possible that in these experiments, the eggs were not exactly under anaërobic conditions but under low oxygen pressures. Loeb's experiment has been repeated, where purified nitrogen was passed through the vessel for a period of twenty-four hours. The eggs were then transferred to a flat-bottomed dish and fertilized with fresh sperm. There were many cytolyzed cells, and on fertilization the eggs gave only 15 to 20 per cent of cell division. Similar experiments performed with fertilized eggs soon after fertilization (the vessels were kept on ice while deaëration was completed) gave identical results, *i.e.*, although there was 100 per cent membrane formation, only 20 per cent of the eggs reached the two-cell stage. Many of these cells gave abnormal types of division. When the eggs are kept in anaërobic conditions for periods of one hour to six hours they can be normally fertilized with fresh sperm (Table I). From experiments on the effect of KCN on the life of sea urchin sperm, Cohn (1918), confirming similar observations of Drzewina and Bohn (1912), concludes that lack of oxygen preserves the life of these cells. When the experiments are performed in *true* anaërobiosis such is not the case. Sea urchin sperm are very sensitive to lack of oxygen. Concentrated sperm suspensions were kept in anaërobiosis for periods of one to five hours. It was observed that they could live, remain motile and fertilize fresh eggs only after one to two hours of anaërobiosis. Three hours later the sperm had lost their vitality and fertilized fresh eggs no more (Table I).

From what has been said about the extreme sensibility of sperm to lack of oxygen it is obvious that fertilization could not be possible when eggs and sperm were kept in anaërobic conditions. Such is the case. Fertilization was performed by introducing the spoon which contained the sperm suspension into the fluid containing the eggs while the vessel was shaken. The nitrogen flow was continued for one hour more. The vessel was opened, and immediately formol was added in sufficient quantity to make a 10 per cent solution. The eggs were examined microscopically and then fixed and stained for later observation.[1] Fertilization occurred in none of these experiments, as tested by the absence of membrane formation. The results

[1] The author wishes to express his thanks to Dr. Fry, who generously had the microscopic sections performed in his laboratory, New York University.

of these experiments are essentially similar to those found by Mrs. Harvey (1930) working at Woods Hole the same year in which these experiments were performed.

Fertilization of anaërobic eggs with fresh sperm was performed by adding a drop of sperm to a burette containing boiled sea water. On account of their great density the sperm go to the bottom of the burette quite readily. The stopcock was opened and four drops of sperm added while the vessel was being shaken.

Anaërobiosis was tested with safranin and by measuring the E. M. F. with a bright platinum electrode. Safranin remained colorless during the whole experiment. The E. M. F. before fertilization was − 0.1805 volts (referred to the H_2 electrode). It remained

TABLE I

The Effect of Anaërobiosis on the Eggs and Sperm of Starfish, Sea Urchin and Nereis, as Tested by Fertilizing Them with either Fresh Sperm or Eggs

Duration of anaërobiosis in hours	Starfish		Sea Urchin		Nereis	
	Eggs	Sperm	Eggs	Sperm	Eggs	Sperm
	Per cent fertilization					
1	90–100	90–100	90–100	90–100	90–100	90–100
2	90–100	90–100	90–100	2	90–100	90–100
3	65–50	50	90–100	None	90–100	90–100
4	None	None	90–100	None	90–100	90–100
5	None	None	90–100	None	90–100	90–100
6			90–100	None		
24			15–20			

constant for 15 minutes, after which it became a little more negative, − 0.1815. During the hour that nitrogen was passing, this E. M. F. rose to −0.184 and remained quite constant. The significance of such a negative potential and its bearing upon the reduction potential of the cell will be the subject of another paper.

Sperm and eggs remained under nitrogen for one hour. The vessels were opened and treated as indicated. A portion was examined immediately for membrane formation and the rest was kept for sectioning and staining. The results obtained were the following. There was membrane formation on the eggs in all experiments up to six hours anaërobiosis. In the stained sections the spermatozoön could be seen inside the egg cytoplasm at different distances from

the membrane, but in no case was there any nuclear change in the ovum, which remained quiescent [2] (Table II).

The Effect of Anaërobiosis on the Eggs and Sperm of Starfish and Fertilization under Anaërobic Conditions

The metabolic changes accompanying the process of fertilization in these eggs are different from those in *Arbacia*. As Loeb and

TABLE II

Fertilization in Anaërobiosis

The eggs of starfish, sea urchin and *Nereis* have been kept in anaërobiosis during the duration of the experiment and been fertilized either with fresh sperm or sperm kept in anaërobiosis.

	Eggs	Sperm		
	Duration of anaërobiosis in hours	Duration of anaërobiosis in hours	Fresh	
Sea urchin.....	1 to 5	1 to 5		No membrane formation.
	1 to 6		fresh	Membrane formation. Spermatozöon has penetrated inside the egg. Ovum quiescent. No nuclear changes.
Starfish.....	1 to 5	1 to 5		No membrane formation.
	1		fresh	3 per cent membrane formation. No nuclear changes.
	2 to 5		fresh	No membrane formation.
Nereis.....	1 to 3	1 to 3		Fertilization process proceeds for 15 minutes, after which it stops.
	1 to 5		fresh	Similar results.

Wasteneys (1912) have shown, no increase in the oxygen consumption is observed. Loeb attributed this to the fact that the egg becomes mature before the entrance of the spermatozoön. While the eggs of the unfertilized sea urchin show very low metabolic activities, the eggs of the starfish possess a high metabolism. It could therefore be predicted that anaërobiosis would be harmful to the starfish egg, which requires the consumption of a great amount of energy for its normal activity. Starfish eggs kept in anaërobiosis are dead four hours after, as tested by the fertilization method.

The sperm of starfish are more resistant to the effect of lack of

[2] The author wishes to express his thanks to Dr. E. E. Just for examining these sections.

oxygen than the sperm of *Arbacia*. They can remain motile and fertilize fresh eggs after three hours of anaërobiosis. They are dead after four hours of anaërobiosis.

Since both the eggs and sperm of starfish are sensitive to lack of oxygen, it is obvious that they are unfit for the study of anaërobic fertilization. When fertilization was performed using anaërobic eggs and anaërobic sperm there was neither membrane formation nor penetration of the spermatozoön. When fertilization was performed with fresh sperm it was found that eggs kept anaërobically for one hour would give three per cent membrane formation, although, as in the case of sea urchins, no nuclear changes were observed in the stained sections. From two to five hours later there was no formation of membrane.

The Effect of Anaërobiosis on the Eggs and Sperm of Nereis and Fertilization under Anaërobic Conditions

The eggs of *Nereis* are the best material for the study of the initial process of fertilization, since the ovum of *Nereis* eggs is inhibited at the end of its period of growth. The germinal vesicle undergoes none of the preparatory stages of maturation unless the egg be fertilized. Fertilization of *Nereis* eggs is similar in its behaviour towards oxygen consumption to starfish, as there is no appreciable increase in the respiration of the egg after fertilization (Barron, 1931). The eggs of *Nereis* behave like those of the sea urchin in their resistance to lack of oxygen. They can be kept in anaërobiosis for periods varying from one to five hours. When fertilized with fresh sperm afterwards, normal fertilization can be observed in every case.

The sperm show the same resistance to lack of oxygen as the eggs. The sperm were kept in anaërobiosis from one to three hours and fifteen minutes. They were afterwards used to fertilize fresh eggs in normal sea water. In every case there was normal fertilization. With anaërobic sperm and anaërobic eggs, the fertilization experiments were followed from one to three hours. No sections were made and the microscopic observations were performed soon after formol fixation. The jelly, which starts to exude from the cytoplasm three to four minutes after fertilization and lasts for ten to twelve minutes, could be seen in the vessels. At the microscope the cells showed the sperm attached to the fertilization cone. Fertilization proceeded normally until the fifteen-minute period and stopped there. Similar results were observed when fresh sperm and anaërobic eggs were used. No nuclear changes were observed.

Discussion

The experiments performed by previous workers on the subject of anaërobic fertilization have dealt with only one kind of cell, namely, sea urchin cells. From the behaviour of sperm and eggs of three different kinds of animals, representing three different types of fertilization, a study which has been reported above, it is seen that in order to solve the problem it is essential to use the eggs and sperm of animals which are insensitive to anaërobiosis. Sea urchins cannot be used because the sperm are sensitive to lack of oxygen. Starfish cannot be employed because the eggs show identical sensitivity. On the other hand, the eggs and sperm of *Nereis* show a remarkable resistance to anaërobiosis, so that even four hours after anaërobiosis either sperm or eggs can fertilize normally when put in contact with fresh eggs or sperm in the presence of air. It has been shown that fertilization of *Nereis* eggs and sperm is initiated in anaërobic conditions and the process continues until the formation of the fertilization cone, a period corresponding to fifteen minutes. The process stops there. No nuclear changes are observed. The same phenomenon occurs in sea urchin eggs and starfish eggs when fertilization is performed with fresh sperm. The fertilization membrane is formed and the spermatozoön enters into the ovum, but no nuclear changes are observed. It can therefore be concluded that the initial process of fertilization, *i.e.*, penetration of the spermatozoön and formation of the fertilization membrane, is possible in strictly anaërobic conditions, provided the two cells which take part in such process have conserved their vitality. The process stops in this stage and no nuclear divisions take place. These findings seem to favor the hypothesis that if some energy is required for the initial process of fertilization it may be obtained through some hydrolytic process, possibly the breakdown of the carbohydrate molecule into lactic acid, which, as is well-known, does not require oxygen utilization.

The breakdown of glycogen into lactic acid produces an amount of energy which, according to Burk (1929), is from one and a half to two times greater than the heat of reaction. The fact that fertilization does not go beyond the entrance of the spermatozoön into the egg protoplasm and no nuclear changes are observed, may be due to inability of the lactic acid formed to be resynthesized into glycogen on account of absence of oxygen.

Conclusions

1. The eggs of sea urchin are insensitive to anaërobiosis for as long as five hours. After this time cytolysis of the eggs begins.

The eggs of starfish are more sensitive to lack of oxygen. Four hours after anaërobiosis the eggs of starfish are incapable of being fertilized. The eggs of *Nereis* can be kept under anaërobic conditions as long as five hours (maximum limit of our experiments) without any injury.

2. The sperm of sea urchin eggs are very sensitive to anaërobiosis. Two hours after anaërobiosis the fertilization power of the sperm is already diminished. Three hours after, the sperm has lost the power to fertilize. The sperm of starfish are less sensitive to anaërobiosis. They lose their fertilization power four hours after anaërobiosis. The sperm of *Nereis* can be kept as long as five hours in anaërobic conditions without any injury of their fertilization power.

3. Since, in order to reach conclusions regarding the possibility of anaërobic fertilization, it is essential that the two cells be insensitive to anaërobiosis, this condition is fulfilled only in the case of *Nereis*. Fertilization in *Nereis* eggs can be started with both eggs and sperm having been kept in anaërobiosis for five hours. The fertilization process is stopped with the formation of the fertilization cone, *i.e.*, which corresponds to fifteen minutes after normal aërobic fertilization has started. No nuclear changes are seen. Fertilization is started in sea urchin eggs when fresh sperm are used. The process stops with the formation of the fertilization membrane and the entrance of the spermatozoön into the egg protoplasm. No nuclear changes take place. In starfish eggs, anaërobic fertilization with fresh sperm is possible to a certain extent (membrane formation and entrance of spermatozoön).

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FAT METABOLISM OF THE CHICK EMBRYO UNDER STANDARD CONDITIONS OF ARTIFICIAL INCUBATION

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The bird's egg consists of about forty per cent of fat. The largest portion of the fat is found in the egg yolk. In addition to this, during incubation period some fat is synthesized from protein and some possibly from carbohydrate molecules. All this fat is utilized for nutrition and energy supply of the developing embryo and of the young bird after hatching.

Among the earliest workers with avian eggs Parke (1866) noticed that the amount of fat in the egg yolk diminishes during incubation. Then Eaves (1910), Sakuragi (1917), Idzumi (1924) and Murray (1926) showed experimentally that as the actual amount of fat decreases in a whole egg, it increases in an embryo. Needham (1925 and 1927) in his reviews of the literature pointed out that the fat in an egg is the most important energy-source of the developing embryo.

The above facts would suggest that the egg fat is of such chemical composition that it can be utilized according to the need of the embryo. The process of absorption of fat, particularly of yolk fat, also gives another aspect of the question. It looks very much as if there is a preferential absorption of the unsaturated acids by the embryo at certain stages of incubation (Eaves, 1910).

The present paper consists of a further experimental study of the fat metabolism of the chick embryo under standardized or "normal" conditions of artificial incubation, that is, changes of the amount and chemical nature of fat in the embryo and egg yolk as measured by the iodine value, saponification number and refractive index.

METHODS AND MATERIALS

All eggs used were from a flock of White Leghorn hens (*Gallus domesticus*). The eggs were selected for uniformity of size and quality of eggshell (Romanoff, 1929).

The incubation was carried on in a special electric laboratory incubator (Romanoff, 1929*a*) under the conditions already described (Romanoff, 1930) with the temperature 38.0 $\pm$ 0.2° C., and the relative humidity 60.0 $\pm$ 1.0 per cent.

TABLE I

Iodine Value, Saponification Number and Refractive Index of Fat (ether extract) of Yolk of Fowl's Egg

Author	Percentage of Fat in Wet Weight	Iodine Value	Saponification Number	Refractive Index
	per cent			$(n)_D$
Parke (1866)	22.82	—	—	—
Amthor and Zink (1897)	—	75.4	189.4	1.4670
Kitt (1897)	19.00	71.1	190.2	—
Peninngton (1909)	32.39	63.6	182.2	1.4611
Serono and Pallazi (1911)	20.17	82.31	198.85	—
Thomson and Sorley (1924)	31.10	74.73	183.79	—
Romanoff (present rpt.)	30.71	73.89	190.69	1.4690

At intervals of 24 hours all eggs were candled for dead embryos, and at least four eggs with normally developed embryos were removed for analysis.

Previous experience in our laboratory showed that at certain stages of embryonic development it is almost impossible to separate the yolk from albumen or allantoic fluid, and to determine it quantitatively with appreciable accuracy. Therefore the boiling of eggs,—to complete coagulation of yolk and albumen,—had been employed. This method proved to be very quick and satisfactory, not only in determining the total values of yolk and albumen, but also in some chemical analysis, such as fat (ether extract) determination.

TABLE II

Distribution of Fat (ether extract) in a Fresh and Hatched Egg

Parts of the Egg	Fat (ether extract)	
	Amount	Percentage
	grams	*per cent*
Fresh egg:		
Yolk	5.8275±1.0971	99.50
Albumen	0.0046±0.0013	0.08
Shell	0.0033±0.0025	0.06
Shell membranes	0.0213±0.0167	0.36
Total	5.8567	100.00
Hatched egg:		
Chick	1.8887±0.1224	48.57
Yolk sac	1.9315±0.0762	49.67
Shell with waste matter	0.0682±0.0126	1.76
Total	3.8884	100.00
Difference (combusted fat)	1.9683	33.61

TABLE III

Changes in the Fat (ether extract) Content of the Chick Embryo

Stage of Incubation	Weight of Embryo			Fat (ether extract) Content			
	Wet Weight	Dry Weight	Percentage of Dry Matter	Composed Samples	Average Fat Content	Percentage of Wet Weight	Percentage of Dry Weight
days	*grams*	*grams*	*per cent*	*embryos*	*grams*	*per cent*	*per cent*
8	1.350	0.099	7.33	18	0.0133	0.99	13.13
9	2.083	0.147	7.06	12	0.0233	1.12	15.85
10	2.700	0.238	8.81	6	0.0310	1.15	13.03
11	3.850	0.305	7.92	6	0.0539	1.40	17.67
12	5.499	0.513	9.33	5	0.1015	1.85	19.79
13	7.213	0.703	9.75	4	0.1610	2.22	22.90
14	9.670	1.159	11.99	2	0.2575	2.66	22.22
15	11.327	1.544	13.63	2	0.3475	3.07	22.51
16	15.360	2.582	16.81	2	0.5012	3.26	19.41
17	18.095	3.396	18.77	2	0.7997	4.42	23.55
18	22.310	4.220	19.92	2	0.9823	4.40	23.28
19	29.891	5.812	19.44	1	1.3024	4.36	22.41
20	31.298	5.999	19.17	1	1.4053	4.49	23.43

TABLE IV

Changes in the Fat (ether extract) Content of the Yolk Sac

Stage of Incubation	Average Egg Weight	Weight of Yolk Sac			Fat (ether extract) Content		
		Wet Weight	Dry Weight	Percentage of Dry Matter	Average Content	Percentage of Wet Weight	Percentage of Dry Weight
days	*grams*	*grams*	*grams*	*per cent*	*grams*	*per cent*	*per cent*
0	61.6	18.44	9.654	52.35	5.6633	30.71	58.66
1	62.0	19.19	10.110	52.68	6.4837	33.79	64.17
2	62.5	19.79	10.113	51.10	6.0309	30.47	59.54
3	62.5	20.10	9.546	47.49	6.0338	30.02	63.21
4	62.5	21.79	10.390	47.68	6.5621	30.12	63.16
5	61.3	20.22	8.781	43.43	5.3549	26.48	60.98
6	61.8	21.51	8.872	41.24	5.4276	25.23	61.18
7	62.3	21.81	9.354	42.88	5.8198	26.68	62.22
8	62.5	20.58	8.611	41.84	5.4874	26.66	63.73
9	60.3	19.84	8.726	43.98	5.6041	28.25	64.22
10	62.0	19.08	8.767	45.95	5.2271	27.39	59.62
11	61.3	18.80	8.382	44.59	5.3328	28.37	63.62
12	61.5	18.27	8.617	47.17	5.4158	29.64	62.85
13	62.0	18.31	9.097	49.68	5.4335	29.68	59.72
14	59.3	16.64	9.539	57.35	5.5897	33.59	58.59
15	62.5	16.34	9.676	59.25	5.4803	33.55	56.64
16	60.0	14.54	9.006	61.94	4.5241	31.11	50.23
17	61.0	14.02	9.132	65.14	4.4922	32.04	49.19
18	62.5	13.87	8.255	59.52	3.0893	22.27	37.42
19	59.5	12.03	6.986	58.57	2.4567	20.42	35.17
20	60.5	10.53	6.015	57.12	2.1434	20.35	35.64

In these experiments two eggs were boiled for 20 minutes, the coagulated yolk was weighed, then dried to constant weight in a Freas electric vacuum oven at 55° C. and vacuum at about 63.5 cm., ground and subjected for 48 hours to extraction of fat with anhydrous ether by a Soxhlet extraction apparatus.

From two or more unboiled eggs the embryos were separated for determination of dry matter and fat content by methods similar to those described above.

The extracted fat from both the embryo and the yolk sac was tested for saponification number (Koettstorfer, 1879), iodine value (Wijs, 1898),[1] and refractive index, by a Zeiss butter refractometer.

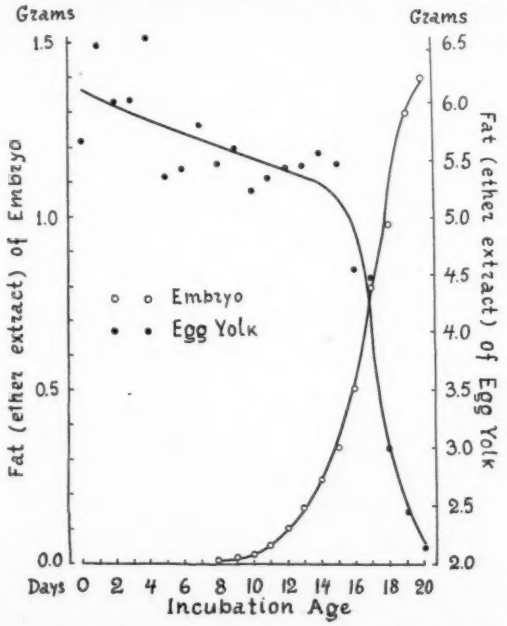

FIG. 1. Changes in the fat (ether extract) content of the embryo and egg yolk during incubation.

RESULTS AND DISCUSSIONS

It has been found that the fat content of a fresh fowl's egg is directly proportional to its size. For that reason the amounts of fat obtained from an egg by previous workers can hardly be compared,

[1] Both saponification number and iodine value were determined after the methods given in *Official and Tentative Methods of Analysis of the Association of Official Agricultural Chemists*, third edition, 1930, on pp. 321–322.

unless the data are expressed in percentages of dry or wet weight of the substance. Similar inconsistency is observed in the data on iodine value, saponification number and refractive index of the fat from an egg yolk (Table I). These dissimilarities might be accounted for by the lack of uniformity in material and methods, such as: the method of extraction, care of the extract, age of eggs and possibly seasonal quality of eggs.

It is evident from the data of Table II that the egg yolk is the main source of fat to the developing embryo. In a fresh egg there is

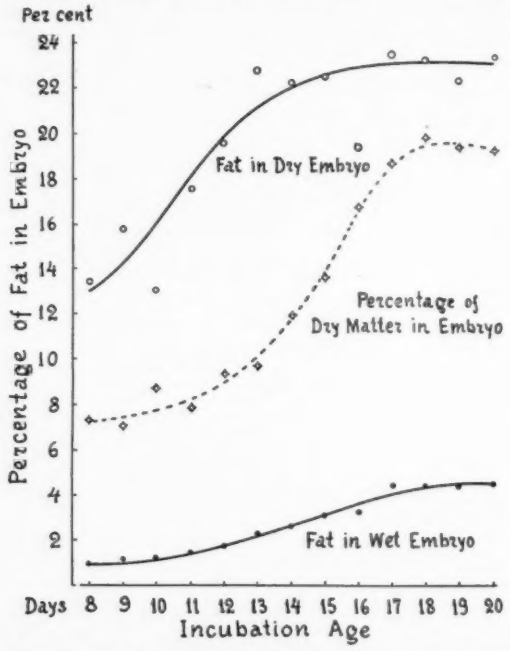

FIG. 2. Percentages of fat (ether extract) in the wet and dry weights of the embryo at various stages of incubation. (The curves of the above values are not identical due to changes in the dry matter content of the embryo, which is shown by dotted line.)

about 99.5 per cent of yolk fat and only 0.5 per cent of fat in the remainder of the egg, including albumen and shell with shell membranes. At the time of hatching the yolk sac still has the largest portion of reserve fat for the nutrition of the chick after hatching. Next in amount is the fat absorbed by the embryo. Then a small amount of fat is retained in the waste matter and in the shell with

shell membranes. The combusted fat represents 33.61 per cent, or perhaps a larger amount if synthesis of fat had taken place during the incubation.

The daily fat content of the developing embryo and egg yolk (Tables III and IV) gives a general idea of the changes which go on within an egg during incubation (Fig. 1). The fat of the embryo increases noticeably only after about two weeks. The fat of the yolk at first decreases slowly; then it rapidly falls from about the sixteenth day up to hatching time.

The curves on the percentages of fat in the wet and dry embryo (Fig. 2) are not identical. Change in the moisture content of the embryo throughout the incubation period is the main influencing factor. Therefore a true percentage value should be taken from dry rather than wet weight of the embryo.

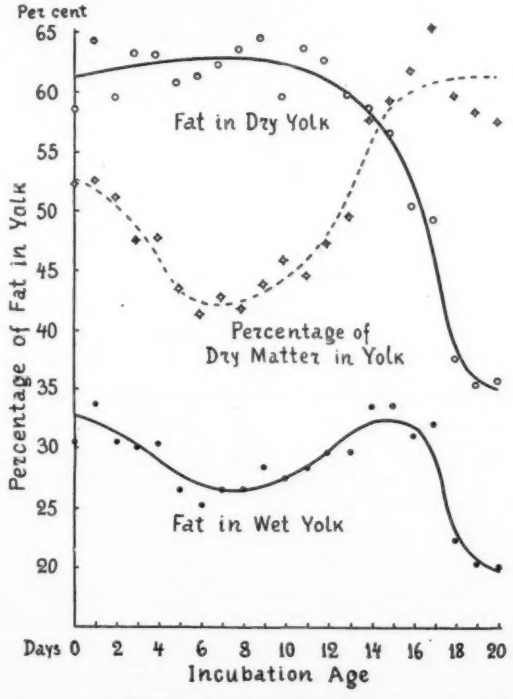

FIG. 3. Percentages of fat (ether extract) in the wet and dry weights of the egg yolk at various stages of incubation. (The curves of the above values are not identical due to changes in the dry matter content of the yolk, which is shown by dotted line.)

The influence of the moisture content of the egg yolk on the curves of the percentages of fat from wet and dry weight (Fig. 3) is still greater than the influence of the moisture content of the embryo. The curve of the percentage of fat in dry yolk is the only one which demonstrates regularity in changes of fat during incubation. This curve shows that the percentage of fat in yolk is increasing somewhat during the first week, declining during the second and dropping during the third week. The rise of the curve may be due to synthesis of fat from other chemical substances; the decline due to the noticeable

TABLE V

Refractive Index of Fat from the Chick Embryo and Yolk Sac

Stage of Incubation	Refractive Index $[(n)_D]$	
	Embryo Fat	Yolk Fat
days		
0	—	1.4685
9	1.4879	—
10	[1.4903]	—
11	1.4877	—
12	1.4841	—
13	1.4844	1.4687
14	1.4816	1.4695
15	1.4758	1.4693
16	1.4763	1.4695
17	1.4741	1.4675
18	1.4735	1.4691
19	1.4727	1.4696
20	1.4719	1.4690

growth of the embryo; and the drop due to the rapid and sole utilization of fat for growth and for energy supply to the embryo.

The combusted fat accompanying embryonic development, is the expression of the energy expended. It has been observed that the greatest part of the egg fat is used toward the end of hatching time. The curve of the combusted fat would be very similar to the curve of the growth of the embryo (Romanoff, 1930).

The extracted fat from egg yolk throughout the incubation period shows hardly any change in the saponification and iodine numbers and refractive index, giving on an average 73.89, 190.69, and 1.4690 respectively. On the other hand, there was found a pronounced change in the iodine number and refractive index of the fat from the embryo (Table V, Fig. 4). The iodine number of the fat from the embryo at 16 days of incubation was 78.84; on the following days it

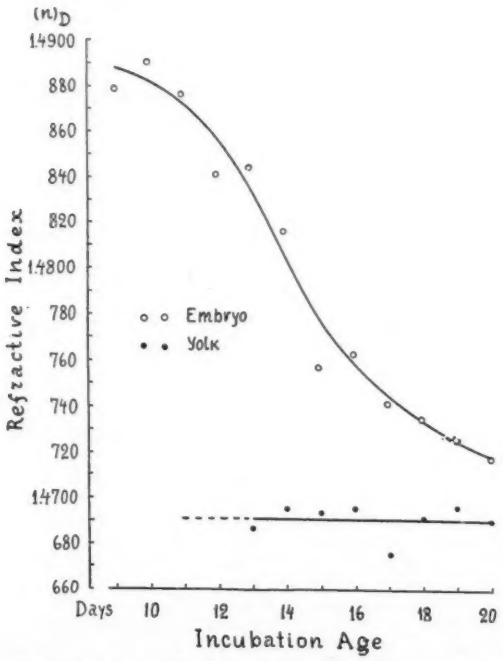

FIG. 4. Refractive index of the fat from the embryo and egg yolk during incubation.

was 82.03, 83.69, 84.03, 88.20, reaching 90.34 at the time of hatching. The refractive index was found to be high at the beginning of observation, nine days of embryonic age, and was rapidly decreasing towards hatching.

The author wishes to express his thanks to H. A. Faber for assistance in routine analytical work covering this paper.

SUMMARY

1. Fat content of fresh eggs is a subject of great variation, due primarily to disparity in size of individual eggs.

2. Iodine value, saponification number and refractive index of the fat from fresh eggs are rather constant, but only under a given experimental condition.

3. The main source of fat in an egg is the egg yolk, which furnishes the fat for nutrition and energy-supply of the developing embryo.

4. The amounts of fat in the growing embryo and egg yolk give a comprehensive idea about the changes in fat which go on within an egg during incubation.

5. The relative increase of fat in the embryo and the decrease of it in the yolk can be well demonstrated by the curves plotted from the data on the percentages of fat in dry weight.

6. The curve of combusted fat is quite similar to the curve of the growth of embryo.

7. Iodine value, saponification number and refractive index of the fat from the yolk sac of the developing egg are almost constant through the incubation period.

8. Iodine value and refractive index of the fat from the developing embryo are increasing and decreasing respectively during the latter part of incubation.

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IS THE ERYTHROCYTE PERMEABLE TO HYDROGEN IONS?

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I

The erythrocyte is generally believed to differ from most other cells in the ease with which its internal reaction is influenced by that of its surroundings. Even in the absence of so-called "penetrating" acids and bases such as CO_2, fatty acids, ammonia, etc., which alone are effective with ordinary cells, it is easy to bring about in it striking internal pH changes. The mere hydrogen ion concentration (strictly speaking, the hydrogen ion activity) of the surrounding medium, regardless of how it is produced, seems automatically to determine in the erythrocyte an internal hydrogen ion concentration that can be predicted by the principles of the Donnan equilibrium (Warburg, 1922; Van Slyke, Wu and McLean, 1923). This interrelation of external and internal pH is part of an important mechanism for preserving an approximately constant blood reaction under all ordinary physiological conditions.

While the establishment of a Donnan equilibrium between the erythrocyte and its surroundings is almost universally considered to be due to a passage of ions across the cell membrane, there has been some doubt as to the particular ions involved in the case of pH changes. Partly, no doubt, owing to our customary methods of measuring and defining the reactions of aqueous solutions, and partly to the fact that physiologists, in general, have had to deal more frequently with the penetration of cells by acids than by alkalies, it has been customary in the past to postulate a ready permeability of the erythrocyte to hydrogen ions. But it has been pointed out by Van Slyke, Wu and McLean (1923) and others that exactly the same end results would be obtained if the permeability were to hydroxyl rather than to hydrogen ions, since, as is well known, the relation existing between these two ions in aqueous solutions is such that the activity of the one is related in a fixed manner to that of the other.

In cases where our interest is primarily in equilibria, it is a matter of indifference whether the cell is permeable to the hydrogen or to the hydroxyl ion, or to both; indeed, by the study of equilibrium

states alone it is impossible to reach any decision as to the mechanism by which these states have been reached. Since most of the work heretofore done on the erythrocyte has been concerned with equilibria, it is not surprising that the question of the relative penetrating powers of hydrogen and hydroxyl ions has received almost no attention. However, this question is obviously one of considerable importance to those interested primarily in the mechanism of cell permeability. The erythrocyte, as is well-known, appears to be permeable to anions and impermeable to cations (for a summary of the evidence see Jacobs, 1931), and a single exception to the general rule would make necessary a more complicated theory of ionic permeability than would otherwise be required. It is true that the hydrogen ion is unique in several other respects, and it is by no means inconceivable that it might be in its powers of penetrating the erythrocyte as well. It would, however, considerably simplify the situation if direct evidence could be furnished in favor of the other alternative which is, *a priori*, the more probable of the two.

In the present paper evidence of this sort has been obtained by the use of a simple method, which may, incidentally, prove to be useful in the investigation of other problems connected with the physiology of the erythrocyte. Though this evidence is not presented as conclusive proof of the view that pH adjustments between the erythrocyte and its surroundings are produced through the agency of the hydroxyl ion, it is believed that the observed facts may be explained more simply in this way than in any other; indeed, we have been unable to find any other simple and plausible explanation that even remotely fits the facts.

II

One of the most convenient methods for studying permeability to hydrogen and hydroxyl ions is to employ cells which contain natural indicators of some sort whose color is affected by changes in intracellular reaction. It happens that the erythrocyte belongs in the category of cells showing such color changes. As is well known, hemoglobin, in the presence of sufficiently high concentrations of acids, loses its red color and becomes converted into brownish, or in dilute solutions, yellowish acid hematin. The fact that this change is irreversible and is not associated with a definite pH value prevents it from being employed in exactly the same way as those of true indicators; but it may, nevertheless, when used with judgment, be of considerable usefulness. In the case of hemolyzed blood in which intimate contact of the acid with the hemoglobin is immediately insured, the color change in the presence of 0.15 M NaCl occurs

practically instantly at pH values of 2.5 or lower; at pH 3.5 the time required may be several minutes and at pH 4.0 may be measured in hours. As far as can be determined by the rather crude methods available, there is no sharp upper limit at which the change entirely ceases.

The same color change occurs, but much more slowly, in the case of suspensions of unhemolyzed erythrocytes; and it is this fact that renders it useful as an indicator of the penetration of acid into the cells. Since, unfortunately, it does not take place sharply at any fixed pH value, it is not a highly accurate means of measuring acid penetration; but within certain limits it appears to be capable of being employed as at least an approximate measure of the rate of this process. The color changes with intact erythrocytes are naturally most conspicuous when the cells are sufficiently numerous to give a distinctly red color to the suspension. A mixture of 1 part of blood to 250–500 of solution (1 or 2 drops to 25 cc.) is that which we have found most suitable; with greater dilutions the color of the suspension is yellowish to begin with and the color change consequently less distinct; with higher concentrations of blood the effect on the pH of the solutions is too great. The color change may be made considerably more striking by first treating the erythrocyte suspension with a trace of carbon monoxide, but to avoid the possibility of unknown complications this procedure was not employed in the present experiments.

Under most conditions which bring about a change in the color of the cells, hemolysis also occurs. The question therefore arises whether the acid penetrates the cells and acts upon the hemoglobin within them or whether the reaction is rather an extracellular one with hemoglobin first liberated by hemolysis. We believe that the former alternative is probably the correct one, for two reasons. In the first place, careful observations have been made, by the method of Jacobs (1930*b*), of the time at which the first traces of hemolysis appear. This proves almost invariably to be after rather than before a distinct color change has occurred (see Tables I, II and III). In the second place, it has been found that within a certain pH range the presence of Na_2SO_4 in proper amounts may either prevent hemolysis almost completely or at least delay it for an hour or more without at all slowing the change of color. Indeed, by starting with a 10 per cent saccharose solution containing 0.015 N HCl, the addition of Na_2SO_4 to a concentration of 0.05 M may considerably accelerate the color change, while strongly retarding hemolysis. In one experiment the following figures were obtained:

	Color Change	Hemolysis
Saccharose alone	285 seconds	390 seconds
Saccharose + Na_2SO_4	165 seconds	>1 hour

The probable reason for the accelerating effect of Na_2SO_4 on the color change will be discussed below. That for the inhibition of hemolysis is not certainly known, but at all events such experiments furnish a clear demonstration of the possibility of intracellular color changes.

Though liberation of hemoglobin from the cells is not necessary in order that the reaction may occur, it must not be thought that the entrance of acid is not influenced by injury to the cells. In observing the behavior of a given suspension, it may be noted that the color change does not proceed gradually and fairly regularly, as it does in the case of hemolyzed blood at sufficiently high pH values, but that for a time the change is extremely slow or entirely invisible, and then suddenly becomes much more rapid, as if a barrier of some sort had been broken down by injury to the cell. Shortly after this point, hemolysis occurs. As contrasted with the slow rate at which the color change is produced by HCl, that in the presence of so-called "penetrating" acids, such as acetic or butyric acids at the same or even considerably less acid pH values, is strikingly rapid. In this respect the behavior of the erythrocyte is similar to that of ordinary cells containing natural or artificially introduced indicators.

In the experiments here reported the erythrocytes were obtained from defibrinated ox blood. The cells were not "washed," since it was believed to be less serious to introduce into the solutions employed the slight traces of blood proteins unavoidably present in dilutions of 1 : 250 or 1 : 500 than perhaps to change the fundamental properties of the erythrocytes in the manner described by Kerr (1929) by previously removing these proteins. The pH determinations were in all cases made with the quinhydrone electrode at the conclusion of each set of experiments. Though the addition of the blood somewhat reduced the acidity of the original solutions, the latter were present in such excess that the pH changes in most cases did not amount to more than a few tenths, or at the highest concentrations of acid, a few hundredths of a pH unit.

The color changes were determined in test tubes by eye, the time given being that at which a distinct color difference could be detected between the experimental tube and an appropriate control. Though it was found that a certain degree of refinement could be introduced by the use of a colorimeter, the advantages of this instrument for the present purposes were not sufficient to compensate for the considerably

greater time required with it to carry out a series of experiments. The determinations of the time of hemolysis, as has already been mentioned, were made by the method of Jacobs (1930*b*), of necessity on a separate, but as nearly as possible identical, suspension of the same blood in the same solution. The few cases in which hemolysis appeared slightly to precede the color change are probably to be accounted for by the fact that the figures were obtained not from a single experiment but from two parallel experiments.

III

The method described in the preceding section was first employed by us in an attempt to throw further light upon an earlier observation (Jacobs, 1930*a*) that the rate of acid hemolysis is greatly influenced by the salt concentration of the external medium. Illustrations of

TABLE I

Effect on time of color change and of hemolysis of adding different amounts of NaCl to 0.02 M HCl in 0.3 M saccharose. All times are in seconds.

Concentration of NaCl	Color change	Beginning of hemolysis	75 per cent hemolysis
—	116	130	153
0.0002	118	127	147
0.0004	108	128	135
0.0008	104	—	—
0.0016	110	—	—
0.0031	116	—	—
0.0063	111	123	137
0.0125	102	111	125
0.025	87	95	119
0.05	71	80	92
0.1	60	71	81
0.2	45	72	80

this effect will be found in Table I and in Tables II and III which, taken together, show the same thing.

The more rapid rate of acid hemolysis in the presence of NaCl might conceivably be due to a more ready penetration of the cells by the acid; on the other hand, since hemolysis is a complicated process, it is also possible that the salt might accelerate the destruction of the erythrocyte in some other way. It was in an attempt to decide between these two possibilities that advantage was taken of a method by which the penetration of the acid could be made directly visible. It was thought that in the presence of different concentrations of salt, a parallel between the time of color change and of hemolysis, with the

color change preceding hemolysis, would indicate an effect of the salt on the actual rate of penetration of the acid. That such a parallel does in fact exist is shown by the experiments to which reference has already been made. In Table I, for example, it will be noted that concentrations of NaCl less than about 0.01 M have little or no effect on either process, but that at higher concentrations the acceleration of hemolysis very closely follows that of the color change.

The question now arises why the entrance of acid and the onset of hemolysis occur more quickly in the presence of NaCl than in its absence. Several conceivable explanations may immediately be dismissed as being decidedly improbable. For example, the case

TABLE II

Time (in seconds except where otherwise indicated) of Color Change and of Hemolysis with Different Concentrations of HCl in 0.15 M NaCl

Concentration of HCl	Color change (hemolyzed blood)	Color change (unhemolyzed blood)	Beginning of hemolysis	75 per cent hemolysis	pH after hemolysis
0.08 N	Almost instantaneous	8	12	25	1.26
0.04	Almost instantaneous	25	29	35	1.55
0.02	Almost instantaneous	39	39	50	1.95
0.016	Almost instantaneous	42	33	60	1.95
0.008	Almost instantaneous	46	44	90	2.25
0.004	Almost instantaneous	53	60	145	2.61
0.002	3	57	72	194	3.06
0.001	9	72	75	232	3.78
0.0005	135	480	67(?)	232	4.62
0.0001	3–4 hours	∞	∞	∞	—

presents superficial analogies with the increased permeability of various cells in the presence of NaCl (Harvey, 1911; Osterhout, 1911, 1922, etc.); but it is easy to show that the accelerated entrance of acid into the erythrocyte and the subsequent hemolysis occur equally readily in the presence of pure isotonic $CaCl_2$ or of physiologically balanced mixtures of NaCl and $CaCl_2$. It has, in fact, been found that a considerable variety of salts are, without exception, effective in facilitating the entrance of HCl into the erythrocyte, though, as mentioned above, sulfates in certain concentrations may inhibit the subsequent hemolysis. It is very improbable, therefore, that there is any close connection between this phenomenon and the older

observations on the increase of permeability produced by sodium salts, which is a specific effect peculiar to these and perhaps a few other salts, and which is readily antagonized by calcium.

Another explanation, applying specifically to the erythrocyte, was next considered, namely, that in the presence of salts some constituent of the cell surface might be removed, thus rendering the cell more permeable to ions. In this connection reference may be made to the work of Brinkman and van Dam (1920), who have reported that it is easy to remove lecithin from the erythrocyte by solutions of electrolytes but not by those of non-electrolytes. An explanation of this sort was, however, rendered very improbable by a simple experiment

TABLE III

Time (in seconds except where otherwise indicated) of Color Change and of Hemolysis with Different Concentrations of HCl in 0.3 M Saccharose

Concentration of HCl	Color change (hemolyzed blood)	Color change (unhemolyzed blood)	Beginning of hemolysis	75 per cent hemolysis	pH after hemolysis
0.32 N	Almost instantly	12	31	52	0.82
0.16	Almost instantly	15	24	29	1.03
0.08	Almost instantly	25	30	36	1.29
0.04	Almost instantly	56	59	69	1.50
0.02	Almost instantly	90	132	144	1.70
0.01	Almost instantly	185	231	269	2.04
0.005	3	270	330	450	2.26
0.0025	75	720	960	—	2.56
0.0012	240	3 hours	4 hours	—	2.87
0.0006	2 hours	5 hours	—	—	3.30

which consisted in comparing the effectiveness of previous washings of the cells with isotonic NaCl solutions, with the actual presence of small quantities of the salt at the time of hemolysis. In one such experiment it was found that ox erythrocytes were no more readily hemolyzed in 0.3 M glycerol containing 0.015 N HCl after six previous washings in isotonic NaCl than before. If the observed effect were merely due to the removal of something from the cell surface, six washings ought to have been far more effective than was the presence in the solution at the time of hemolysis of as low a concentration of NaCl as 0.01 M or less; but this was not the case. From this and similar experiments the conclusion was drawn that it is necessary for

the salt actually to be present with the HCl in order to exert its characteristic influence.

In an attempt to throw further light upon this question, systematic observations were made over a considerable range of acid concentrations both in the presence of NaCl (0.154 M) and in its absence, using for the latter purpose a 0.3 M solution of saccharose. Two such experiments, made on the same blood under as nearly as possible comparable conditions (except for a slight accidental difference, not believed to be significant, in the concentration of the blood in a few of the individual experiments) are summarized in Tables II and III.

The data contained in Tables II and III, which were obtained before the subject had received any theoretical treatment, seemed at first sight somewhat puzzling; but, as will be shown, they have proved to be capable of a very simple semi-quantitative explanation on the basis of a hypothetical permeability of the erythrocyte to hydroxyl rather than to hydrogen ions. It will be noted that in the experiments in question, as in previous ones, color changes and hemolysis always occurred more rapidly in the presence than in the absence of NaCl, other conditions being the same. But not only were the rates of color change and of hemolysis slower in solutions of saccharose than in those of NaCl but in the absence of salt both of these processes entirely failed to occur at concentrations of acid that were otherwise always effective. In other words, it would appear that the salt, in addition to its other effects, influences the position of final equilibrium of the system.

Another difference between the experiments represented in Tables II and III, whose meaning was at first far from clear but which we now believe to be of considerable theoretical significance, is that whereas in the non-electrolyte solutions, over a fairly wide range, the rate of color change and of hemolysis is roughly proportional to the concentration of acid (*i.e.*, doubling the concentration of acid approximately halves the time required for the attainment of the chosen end-point), in the NaCl solutions the concentration of acid is of much less importance. Thus, a forty-fold change in concentration (from 0.001 N to 0.04 N) is seen in Table II to decrease the time required for the color change only from 72 to 25 seconds and for the beginning of hemolysis from 75 to 29 seconds.

In an attempt to account for these various observations, we turned to a consideration of the conditions governing ionic exchanges of various sorts between the erythrocyte and its surroundings. Not only has this treatment of the problem furnished a plausible and simple explanation for all the facts mentioned above, but it has, in

addition, apparently thrown some light upon the equally important question of the relative permeability of the erythrocyte to hydrogen and to hydroxyl ions.

IV

The passage of ions across a membrane can occur only in such a way that electrical neutrality is at all times preserved. Thus, Cl', an ion which is known to pass readily between the erythrocyte and its surroundings, might do so by being exchanged for another univalent anion such as HCO'_3 or OH' or, if the cell were permeable to $H^{\cdot}$ ions, it might cross the membrane in company with one of these cations. The absolute rate of movement of pairs of ions, either in the same or in opposite directions, is not known, but in any given case, by an obvious application of the mass law, the rate of total movement at a given instant ought to be proportional to the product of the concentrations (or activities) of the two members of the pair. When the final equilibrium is reached the total movements in the two directions must balance, giving, therefore, as the condition for equilibrium either:

$$[H]_{solution} \times [Cl]_{solution} = [H]_{cell} \times [Cl]_{cell}$$

or

$$[OH]_{cell} \times [Cl]_{solution} = [OH]_{solution} \times [Cl]_{cell}$$

as the case may be. These expressions are identical with those obtained by Donnan by more rigorous thermodynamic reasoning.

In the case under consideration, it is unfortunately impossible to follow the entire course of the diffusion process by which the final theoretical ionic equilibrium tends to be established. Indeed, in a case where a cell is being hemolyzed and its hemoglobin is at the same time undergoing a fundamental chemical change, anything like an equilibrium is unthinkable. The only part of the process, therefore, about which we can form a reasonably accurate conception is its initial stage when the cell and the surrounding medium both have known compositions. Though information limited merely to this stage is less than we might desire, it is better than none at all; and, indeed, it seems reasonable to suppose that the conditions that determine the initial rate of ionic exchange would exert a similar influence over much of the remainder of the process. It will therefore be instructive to calculate the values of the initial mass law effect for several different types of solutions.

In Fig. 1 are represented the concentrations (which will here be employed as an approximation in place of the more accurate activities, and which are expressed in mols per kilo of water) of certain ions at the instant when a normal erythrocyte is placed in a given solution

FIG. 1

Cell	Solution
(8×10^{-2}) Hb′	
(16×10^{-2}) K·	Na· (y)
(8×10^{-2}) Cl′	Cl′ $\left(x+y-\frac{10^{-14}}{x}\right)$
(5×10^{-8}) H·	H· (x)
(2×10^{-7}) OH′	OH′ $\left(\frac{10^{-14}}{x}\right)$

containing as its only salt NaCl and as its only acid HCl. The pH of the interior of the erythrocyte has been taken as 7.3 and for simplicity all of its salts are represented as KCl. The concentrations given for the cell are merely roughly approximate values for arterial blood (see Henderson, 1928, page 196), since for our present purposes, where only the order of magnitude of the results is significant, a higher degree of accuracy would be meaningless.

As regards the external solution, we have two necessary conditions:

$$[H] \times [OH] = K_w = \text{approximately } 10^{-14}$$

and

$$[Cl] + [OH] = [H] + [Na].$$

Knowing [H] and [Na] which are here represented by x and y, respectively, the values of [Cl] and [OH] are immediately determined by the equations just given. In cases involving sums (but not products) of ionic concentrations in acid solutions, [OH] is usually so small that it may be neglected.

Under the conditions represented in Fig. 1, the directions and initial relative rates of ionic movement, for the two possible methods of pH change, would be determined by:

$$[H]_{solution} \times [Cl]_{solution} - [H]_{cell} \times [Cl]_{cell}$$

and

$$[OH]_{cell} \times [Cl]_{solution} - [OH]_{solution} \times [Cl]_{cell}$$

respectively. Substituting in these equations the various concentrations given in Fig. 1, we have:

$$x^2 + xy - 10^{-14} - 4 \times 10^{-9} \tag{1}$$

and

$$(2 \times 10^{-7})\left(x + y - \frac{10^{-14}}{x}\right) - \frac{8 \times 10^{-16}}{x}, \tag{2}$$

from which we may calculate, as has been done in Table IV, the relative tendencies for the erythrocyte to become more acid (or less

alkaline) in solutions of different degrees of acidity in the presence of an approximately physiological concentration (0.16 M) of NaCl and in its absence.

It is, of course, obvious that the figures in this table, taken singly, have no very great significance; and it is not even permissible at a given pH value to compare with each other the figures for hydrogen and for hydroxyl ions, since the actual rates of penetration of the cell by these ions will depend not merely on the mass law factors but upon the specific properties of the individual ions as well. But for each kind of ion separately over a series of different pH values, the figures have an important relative significance to which attention may now be directed.

It will be noted in Table IV that according to the hypothes's of

TABLE IV

Mass law effect with 0.16 M NaCl and isotonic saccharose solutions at different pH values, for movement across the cell membrane (a) of H· with Cl′ and (b) of OH′ in exchange for Cl′. Internal conditions as described in the text.

pH	0.16 M NaCl		Isotonic saccharose	
	H· with Cl′	OH′ in exchange for Cl′	H· with Cl′	OH′ in exchange for Cl′
7.0	12.00×10^{-9}	24.00×10^{-9}	-4.00×10^{-9}	-8.00×10^{-9}
6.0	15.60×10^{-8}	31.20×10^{-9}	-4.00×10^{-9}	-8.00×10^{-10}
5.0	15.96×10^{-7}	31.92×10^{-9}	-3.99×10^{-9}	-7.80×10^{-11}
4.0	16.01×10^{-6}	32.01×10^{-9}	6.00×10^{-9}	1.20×10^{-11}
3.0	16.10×10^{-5}	32.20×10^{-9}	9.96×10^{-7}	1.99×10^{-10}
2.0	17.00×10^{-4}	34.00×10^{-9}	1.00×10^{-4}	2.00×10^{-9}
1.0	26.00×10^{-3}	52.00×10^{-9}	1.00×10^{-2}	2.00×10^{-8}

permeability to hydrogen ions, the initial mass law factor, for the concentrations of acid actually employed in the experiments, should increase in the sodium chloride solution somewhat more rapidly than the concentration of hydrogen ions. In the saccharose solution, on the other hand, over the same range of concentrations the increase should be approximately proportional to the square of the hydrogen ion concentration. An examination of Tables II and III shows that in the salt solution the observed rate of penetration of the acid is only slightly affected by its own concentration, while in the saccharose solution it is roughly proportional to the first rather than to the second power of its concentration. Evidently the observed facts are in complete disagreement with the hypothesis of permeability to hydrogen ions.

On the other hand, both in the presence and the absence of NaCl

the rate of color change and of hemolysis are in good semi-quantitative agreement with the predicted mass law effects according to the hypothesis of permeability to hydroxyl ions. It is difficult to believe that this agreement is merely the result of chance. Admitting our ignorance of all but the probable beginning of the diffusion process, and making due allowance for the complicating effects of injury to the cells, especially in the more acid solutions, and the very rough nature of the calculations where so many simplifying assumptions have been made, it is nevertheless true that no other explanation of the facts has as yet been found which is at the same time so simple and so well in agreement with the other known properties of the erythrocyte.

The entire failure of color changes and of hemolysis to occur in sugar solutions at acid concentrations which readily bring them about in the presence of NaCl is a necessary consequence of the general theory of ionic exchanges. It will be noted in Table IV that for sugar solutions of pH 7.0, 6.0 and 5.0 the mass law factors for both mechanisms of ionic exchange have negative signs. That is to say, in such solutions the total movement of hydrogen or of hydroxyl ions, as the case may be, must be in the opposite direction from that found in the remaining solutions. In other words, erythrocytes in such solutions should theoretically become more alkaline rather than more acid.

This same problem may also be approached in a slightly different way by introducing the idea of a final equilibrium. Though it is obviously impossible, because of imperfect knowledge of the behavior of hemoglobin and of the erythrocyte at decidedly acid reactions, to calculate the final equilibrium conditions in cells exposed to any desired acid solution, it is nevertheless possible to determine what external solution would cause no internal pH change in normal cells. For the simplified erythrocyte already dealt with, either equation (1) or equation (2) (omitting quantities which are negligibly small) yields the following equation for equilibrium:

$$x^2 + xy = 4 \times 10^{-9}.$$

Solving for x, we have as the external hydrogen ion concentration in equilibrium with an internal pH value of 7.3:

$$x = \frac{\sqrt{y^2 + 1.6 \times 10^{-8}} - y}{2}. \qquad (3)$$

By means of this equation the pH values in Table V have been calculated.

It will be noted in this table that in the entire absence of salt the pH of equilibrium is 4.2, and therefore any solution less acid than this ought theoretically to cause the simplified erythrocyte to become more alkaline rather than more acid. For a concentration of NaCl of 0.0001 M, the critical pH is 4.5, and for one of 0.001, 5.4, etc. These figures, of course, cannot be expected to hold exactly in the case of actual erythrocytes where conditions are more complicated than those here considered. But the general principle itself, to which attention has already been directed by Netter (1928) and which has been put to a practical use by Bruch and Netter (1930), appears to be a sound one whose neglect has probably been responsible for considerable confusion in the past in experimental work with the erythrocyte.

TABLE V

External pH in equilibrium with an internal pH of 7.3 with various external concentrations of NaCl. Internal conditions as described in the text.

Concentration of NaCl (*mols. per liter*)	Equilibrium pH
—	4.2
0.0001	4.5
0.001	5.4
0.01	6.4
0.1	7.4

One further point about Tables II and III deserves mention. Not only is the color change of intact cells affected by the presence or absence of electrolytes, but a similar, though less marked, effect is observable in the case of hemolyzed blood. It is possible that we may here be dealing with a case similar to that reported by Adair, Barcroft, and Bock (1921), who found that even in blood hemolyzed by distilled water there is evidence that the cells, though invisible, may still be sufficiently well preserved to produce characteristic effects upon the dissociation curve of hemoglobin. It is not unreasonable, therefore, to expect that even in hemolyzed blood there might be some evidence of the same salt-acid effect that is found with intact cells. Whether this is a complete explanation of the observed facts, however, or whether some additional principle is involved cannot at present be stated with certainty.

SUMMARY

1. The penetration of acids into mammalian erythrocytes may be followed macroscopically by means of the color changes that occur when hemoglobin is converted into acid hematin.

2. The penetration of the acid precedes, rather than follows,

hemolysis. In certain cases, penetration may be observed without subsequent hemolysis.

3. Over a considerable pH range, both in the presence and in the absence of NaCl, the rate of acid penetration into the erythrocyte, as inferred from the time of color change, is in semi-quantitative agreement with that predicted for a system permeable to hydroxyl and not to hydrogen ions. There is an entire lack of agreement with the theoretical behavior of such a system when the permeability to the two ions is reversed. The simplest explanation of the observed facts is that the hydrogen ion, like other cations, is unable to enter the erythrocyte easily.

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ASCIDIANS OF THE BERMUDAS

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The ascidians of the Bermuda islands have already been described by Van Name (1902) and a revision published by the same author in 1921 under the title "The Ascidian Fauna of the West Indies." In view of this the present paper is confined to a brief account of the fauna as a whole, a description of several new species, and a few notes upon certain other forms.

The ascidians of the Bermudas are almost identical, species for species, with those of the West Indies and Gulf of Mexico, and there is little doubt that, together with the bulk of the Bermuda fauna, they have been carried there from those regions by the west Atlantic drift.

Like ascidians throughout the world, their habitat is determined as a compromise between two factors, their ability to withstand wave-action and exposure, and their need for a good flow of well oxygenated water. They may accordingly be divided into three groups, those to be found only under stones, those attached to the upper surfaces of stones, or to various corals and sea-fans, and those found beyond the outer reefs.

Apart from such classification of habitat further description such as specified localities is practically unnecessary. Species found under stones in one locality are likely to be found in similar positions throughout the islands. The same holds true for forms that are attached to exposed surfaces.

The material upon which this paper is based was obtained through the facilities of the Bermuda Biological Station, St. Georges, and also through the assistance of Louis Mowbray, Esq., of the Government Aquarium.

FAUNA LIST

Aplidium (*Amaroucium*) *bermudae* (Van Name).....on corals
Aplidium (*Amaroucium*) *exile* (Van Name).....under stones
Tridemnum savignii (Herdman).....on algae, under stones
Tridemnum orbiculatum (Van Name).....under stones
Didemnum candidum Savigny.....under stones, on corals
Didemnum (*Polysyncraton*) *amethysteum* (Van Name).....under stones
Leptoclinum macdonaldi (Herdman).....on corals, etc.
Lissoclinum fragile (Van Name).....under stones
Polycitor (*Eudistoma*) *olivaceus* (Van Name).....on stones, corals, etc.

Polycitor (*Eudistoma*) *convexus* (Van Name) ..... on corals, etc.
Polycitor (*Eudistoma*) *clarus* (Van Name) ..... on stones, corals, etc.
Polycitor (*Eudistoma*) *capsulatum* (Van Name) ..... on corals, etc.
Clavelina picta (Verrill) ..... on sea-fans, wrecks, etc.
Clavelina oblonga Herdman ..... under stones, on corals
Cystodytes dellechiaiae (Della Valle) ..... on corals, stones, etc.
Distaplia (*Holozoa*) *bermudensis* (Van Name) ..... under stones, on corals
Perophora viridis Verrill ..... under stones
Perophora bermudensis n.sp. ..... under stones
Ecteinascidia turbinata Herdman ..... on stones, corals, wrecks
Ecteinascidia conklini n.sp. ..... under stones
Ecteinascidia conklini var. *minuta* ..... under stones
Ascidia nigra Savigny ..... on stones, etc.
Ascidia curvata Traustedt ..... under stones
Botryllus schlosseri (Pallas) ..... under stones
Botrylloides niger (Herdman) ..... on and under stones
Symplegma viride Herdman ..... under stones
Polyandrocarpa (*Eusynstyela*) *tincta* (Van Name) ..... under stones
Polycarpa obtecta Traustedt ..... on stones, corals, reefs
Styela partita (Stimpson) ..... on stones, corals, reefs
Pyura vittata (Stimpson) ..... on stones
Microcosmus exasperatus Heller ..... under stones, on reefs

The Bermuda fauna is exceptionally rich in members of the family Perophoridæ. Two well-known forms, *Ecteinascidia turbinata* and *Perophora viridis*, are common. Their close relationship, however, is emphasized by the discovery of transitional types. These new forms may frequently be found growing under the same boulder as those two species.

Inasmuch as the existing descriptions of *Ecteinascidia turbinata* are of immature zoöids, a somewhat detailed account is made here of this species; such an account also forms a basis for comparison of the other forms.

Ecteinascidia turbinata Herdman.

This species was originally described by Herdman from material collected at Bermuda by the Challenger expedition. Large orange colonies develop attached to the upper surfaces of rocks where currents are relatively strong.

The essential organization of the zoöid is shown in Fig. 1. The structures shown here for the first time are the heart, and the gonads with their ducts. The heart is of the same relative size and length, and is in the same relative position as in species of Ascidia or Phallusia. The gonads develop in the intestinal loop on the left side, as in Perophora, the ovary being surrounded by a ring of testicular lobes. The vas deferens during its distal course follows the intestine and they open together some distance short of the atrial siphon. The oviduct, on the other hand, is relatively short and wide. It passes

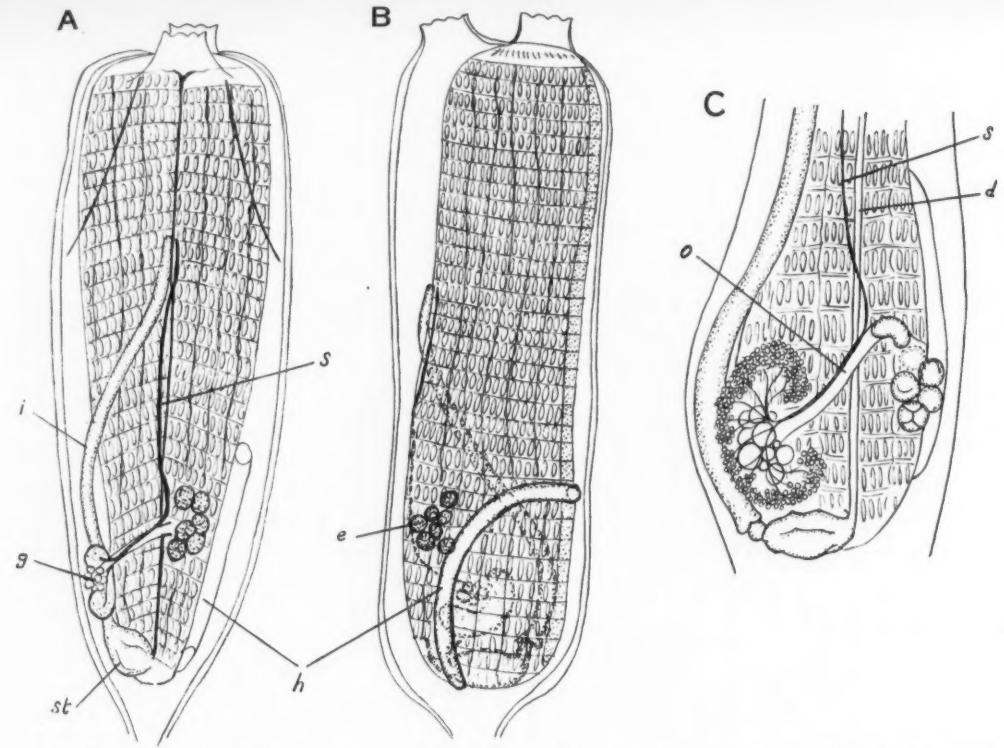

FIG. 1. *Ecteinascidia turbinata* Herdman. Mature blastozoöids, A, from dorsal, B, from right side; C, from dorsal and left aspect. *d*, dorsal lamina; *e*, embryos; *g*, gonads; *h*, heart; *i*, intestine; *o*, oviduct with egg emerging from it into right atrial cavity to join developing embryos; *s*, sperm-duct; *st*, stomach.

from the ovary to the dorsal lamina, opening beyond it into the atrial cavity of the right side. A single egg at a time is forced up the duct. During this brief passage it becomes greatly elongated through compression, rapidly regaining its spherical shape as it emerges.

The above features are generic, the characters of specific importance being as follows,—the tendency to grow on the exposed surfaces of rocks, the red or orange colour, the proximity of the atrial to the branchial siphon, the possession by the adult zoöid of about thirty rows of stigmata, and the formation of tadpole larvæ with twelve rows of stigmata. The breeding season extends from about June first to August or September.

Ecteinascidia conklini n. sp.

This species was at first mistaken for young colonies of *E. turbinata*. The generic features are identical with those of *E. turbinata*. The specific differences are the following: it is found only on the under side of rocks, the colour is green or yellow-green with but a thin red ring around the siphons, sometimes absent; the atrial siphon is long and placed some considerable distance from the branchial siphon; the rows of stigmata in the zoöids average about twenty in number, while smaller tadpoles are formed with but six rows of stigmata; the eggs are smaller, and the breeding season commences about one month later than that of *E. turbinata*, under identical conditions.

Ecteinascidia conklini var. *minuta* n. sp.

This form was found with mixed colonies of *E. conklini typica* and *Perophora viridis*. It differs from the type species in a few minor features only, which nevertheless seem worthy of recording.

It is smaller; the number of rows of stigmata in the zoöid rarely exceeds fifteen; the atrial siphon is short and close to the branchial siphon while both bear prominent ridges, and the eggs and larvæ are smaller although the tadpole possesses six rows of stigmata as in the other. The breeding season as far as is known coincides with that of *E. conklini typica*.

Probably the most definite feature distinguishing both *E. conklini typica* and *minuta* from *E. turbinata* is the curvature of the intestine in the former, tending to form a secondary loop. The intestine of *E. turbinata* is but very slightly curved in comparison.

Perophora bermudensis n. sp.

This species forms mixed colonies with the common *Perophora viridis*, both growing in great profusion on the under surface of stones where there is a good circulation of water.

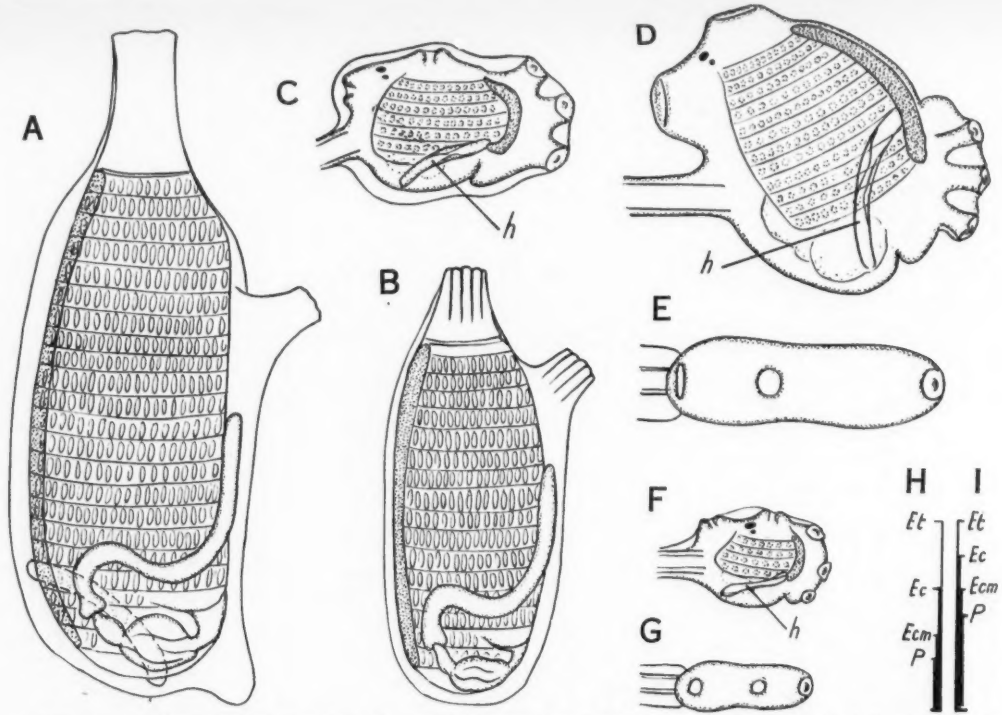

FIG. 2. A, blastozooid of *Ecteinascidia conklini* n. sp., from right side. B, blastozooid of *E. conklini* var. *minuta*, from right side. C, tadpole of *E. conklini* (that of *E. conklini minuta* is identical except for being smaller). D, tadpole of *E. turbinata* from side, E from top. F, tadpole of *Perophora viridis* from side, G from top. H, relative sizes of mature zooids of the three species of Ecteinascidia and of Perophora. I, relative sizes of the eggs of the same forms.

The zooids are drawn all to one and the same scale, while the tadpoles are also drawn to one scale.

h, heart.

Perophora viridis has a greenish tinge, as its name implies, and both oozoöid and blastozoöid have four rows of stigmata. *Perophora listeri* is similar in that the greenish colour is quite absent, but it forms by no means such dense colonies. *Perophora annectens* differs from both these species in that it has but three rows of stigmata.

Perophora bermudensis possesses blastozoöids with five rows of stigmata (although the oözoid has but four) and is accordingly unique. It may be readily distinguished from *P. viridis* with which it is usually entangled, by the absence of any green colour and the somewhat larger size of the zoöids.

Its structure is shown in Fig. 3, and it is seen that not only are there five rows of stigmata but that about a third of the stigmata of

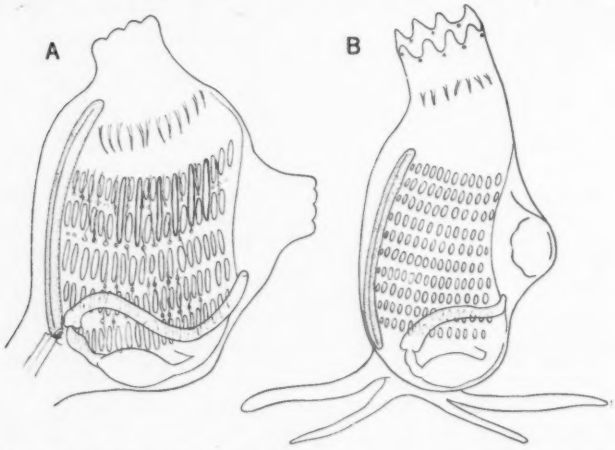

FIG. 3. A, blastozoöid of *Perophora bermudensis* n.sp. B, oozoöid of *Ecteinascidia conklini* or else a blastozoöid of *Perophoropsis herdmani* Lahille.

the first and second rows are common to both. This condition prevails in the smallest and youngest blastozoöids in which stigmata are discernible.

Perophoropsis herdmani Lahille.

This form was described by Lahille in 1890 from Banyuls and has not been rediscovered. It differs from species of *Perophora* in that there are at least ten rows of stigmata. It is a genus therefore intermediate between *Perophora* and *Ecteinascidia*.

At Bermuda, mixed with colonies of *Perophora viridis*, *P. bermudensis*, and *Ecteinascidia conklini*, were found individuals corresponding in structure to *Perophoropsis;* these are shown in Fig. 4.

There is, however, another possibility. It was determined in the case of *Ecteinascidia turbinata*, *E. conklini*, *Perophora bermudensis*, *Clavelina lepadiformis*, *Botryllus gigas*, and *Botrylloides leachii*, that in developing blastozoöids the number of rows of stigmata, while yet non-functional although perforate, equals or may slightly exceed the number of stigmata rows in the zoöid from which they arose. In no case was it less than that number. This statement is probably true for ascidians as a whole and will form the subject of further investigation.

This phenomenon accordingly rules out the possibility that the Perophoropsis-like forms were young blastozoöids of *Ecteinascidia;* while the fact that the oozoöid of *E. turbinata* possesses from the start twelve rows of stigmata makes it impossible that they are young oozoöids of that species. With *E. conklini* the case is different. The oozoöid first becomes active with but six rows of stigmata, and accordingly it is possible that the forms in question are slightly-grown individuals of this type, especially as they were solitary and without budding stolons, although occurring not far from one another. Another feature which supports such a contention is that the individual stigmata are but half the length of those of *Ecteinascidia* or *Perophora* species. Whether they are blastozoöids or oozoöids, they are probably but half-grown.

Provisionally, therefore, these perophorids will be assumed to be young oozoöids of *Ecteinascidia conklini* rather than the *Perophoropsis* of Lahille. If this is the case, then it should be noted that additional rows of stigmata appear in the oozoöid when the stigmata of the original six rows are merely a fourth of full adult size, and cannot be distinguished from those first six rows.

Genus *Clavelina*.

There is some confusion as to the number of species of *Clavelina* to be found in the Bermuda and West Indian region.

From material taken on the Challenger expedition at Bermuda Herdman described a form that he named *Clavelina oblonga*. In 1900 Verrill, in his general account of the fauna of the Bermudas, described an ascidian under the name *Diazona picta*. In the account of the Bermuda ascidians published by Van Name in 1902 this last was changed to *Rhodozona picta* on the ground that it differed materially from the type of *Diazona* and was intermediate in character between *Diazona* and *Clavelina*. In his later account (1921) of the ascidian fauna of the West Indies Van Name includes both these forms under *Clavelina oblonga*, the *Diazona picta* of Verrill being considered to be large colonies and Herdman's *Clavelina oblonga* young colonies of

one and the same species. This he describes as a form in which the zoöids of a young colony are almost completely separated from one another but which in older colonies become enclosed for their greater part in a common gelatinous test.

Such a conclusion is believed to be the result of two factors, the study mainly of preserved material, and of material collected not later than May, that is, of material somewhat immature. Preservation destroys pigmentation and in these two forms results in a tremendous shrinkage of the thoracic part of the zoöids.

These forms are believed to be both clavelinids but also to be quite distinct species. On this assumption then the one should be known as *Clavelina oblonga* Herdman and the other as *Clavelina picta* (Verrill). It is hoped that the following descriptions will show this to be the case.

Clavelina oblonga Herdman.

Habitat.

Attached to the under-surface of stones near low-water level, very rarely in more exposed positions.

Breeding season.

End of April until August.

Form of colony.

Number of zoöids rarely exceeds 40, usually much less. They are attached to a basal stolon, but otherwise are separated from one another. Budding occurs throughout breeding season so that zoöids of all sizes are to be found.

Pigmentation.

Test crystal-clear. Branchial sac with flecks of white pigment near its anterior end. Abdominal region yellowish. In living state whole colony perfectly transparent.

Egg and tadpole size.

0.31 mm. diameter (egg).
2.25 mm. total length (tadpole).

Post-larval development.

Tadpole and young oozoöid have from the first, two rows of definitive stigmata.

Clavelina picta (Verrill).

Habitat.

Attached to sea-fans, corals, submerged wrecks, etc., from low-water level to a few fathoms; never found under stones; a good flow of water seems to be necessary.

Breeding season.

End of June until August or September.

Form of colony.

Colonies usually possess more than 40 zoöids and may have as many as 1000. The zoöids are embedded in a common test, which is usually divided into corms. During the summer the thorax of each zoöid extends beyond the common test, at other times it is usually completely embedded. Budding does not occur during the breeding season or the months preceding it and so the size of the zoöids is very uniform in any one colony.

Pigmentation.

Test unpigmented but slightly opaque. Endostyle, dorsal lamina, and peripharyngeal bands densely coloured with purple or carmine cells. They also extend throughout the abdomen and then accumulate in the ends of the test vessels. They appear in the late embryo in such places and the oozoöid is as highly coloured as the blastozoöids.

Egg and tadpole size.

0.49 mm. diameter (egg).
3.30 mm. total length (tadpole).

Post-larval development.

Tadpole and young oozoöid have from the first, four rows of definitive stigmata. All other species of *Clavelina* have but two.

Clavelina oblonga usually occurs in groups of two or three during May and increases in number throughout the breeding season until about forty zoöids of various size may be found in one colony. This was found to be the case in both 1930 and 1931, and the implication is that the oozoöid forms a few winter statoblasts which regenerate and give rise to the type of colony just described during the following spring, while such a colony dies off at the end of the summer, failing to form a second generation of statoblasts. That is, the life-cycle is completed within 18 months; otherwise, large colonies should be found similar to those of *Clavelina lepadiformis*, which is not the case.

That *Clavelina picta* was originally taken to be a diazonid is due

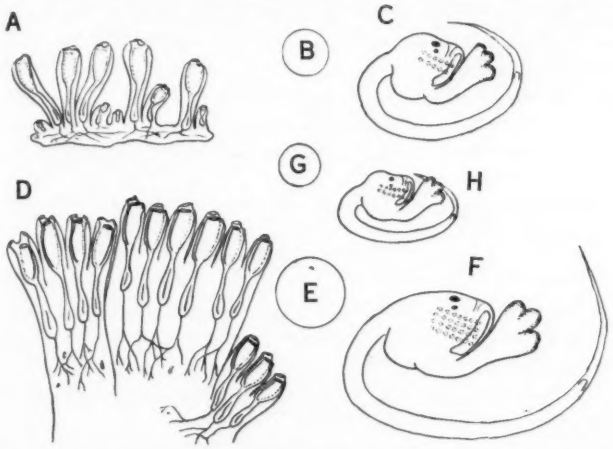

FIG. 4. A, colony of *Clavelina oblonga* Herdman. B, C, egg and tadpole of same. D, part of a colony of *Clavelina picta* (Verrill). E, F, egg and tadpole of same. G, H, egg and tadpole of *Clavelina lepadiformis*.
The eggs and tadpoles are drawn to the same scale.

to a superficial similarity in type of budding. In *Diazona violacea* budding by abdominal strobilation occurs after the breeding season has ended, and only then. So that every autumn each zoöid forms eight or ten compact bodies which regenerate slowly through the winter and spring to become sexually mature in the late summer; the process is then repeated. Therefore the zoöids are uniform in size and remain embedded in the common test, although the anterior part of the thorax of each extends separately during the late summer.

In *Clavelina picta* winter statoblasts are formed after the breeding season and apparently at no other time. The oozoöid probably forms about ten such bodies. These regenerate, grow and become sexually

mature during the winter and spring. They carry on these processes close together within the test of the oozoöid, and so remain within a common test. During the late summer these processes are repeated, resulting in the formation during the following year of a colony composed of about ten corms, each containing ten or more zoöids. This may be repeated a third year to form massive colonies. During maturity the anterior parts of the zoöids extend from the common test just as in *Diazona*, and as in *Diazona* they contract and degenerate after the breeding season while the thoracic extensions of the test are sloughed off. Thus, apart from the difference in budding, strobilation of the abdomen in *Diazona* and the formation of post-abdominal extensions in *Clavelina*, the cycle is much the same in the two genera and it is understandable that a superficial similarity results. The separation of the zoöids in large colonies of *Clavelina lepadiformis* in contrast to the condition in *C. picta* is due probably to the very thin test of the former, so thin that when the statoblasts grow into new individuals they form independent vertical extensions of it from the first.

Budding in these forms will be the subject of a much more detailed investigation.

Symplegma viride Herdman.

This species was described first by Herdman from material collected at Bermuda by the Challenger expedition, and has since been discovered throughout the West Indies, and in the East Indies from the Philippines to the Red Sea. A full description with illustration of a zoöid is given by Van Name (1921).

This form has at various times been included in the Styelidæ and in the Botryllidæ. A few additional observations made at Bermuda merely emphasize its relationship to both these groups. In fact it so completely bridges the gap, both in its adult structure and organization and in its development, between these two families that their maintenance as distinct families is an unnatural classification. It is accordingly proposed that the Botryllidæ be subordinated within the Styelidæ, either as a sub-family or merely as constituent genera.

The method of budding in its fundamentals is identical in the Polystyelidæ, Symplegma, and Botryllidæ.

Symplegma resembles polystyelids such as *Polyandrocarpa*, *Distomus*, or *Stolonica* inasmuch as each zoöid has its own atrial siphon, common cloacal cavities not being formed. Its general anatomy resembles that of *Polyandrocarpa* or *Polycarpa* except that its individuals are much smaller. Correlated with this reduction is a

diminution in number of polycarps (to a single polycarp) and in number of rows of stigmata. Its structure differs from that of botryllids only in the absence of common cloacal cavities, and consequently in the lack of obvious systems in the colony. In other words, common cloacal cavities and the arrangement of zoöids into systems are the only major features that separate the botryllids from other styelids. Apart from these two characters *Symplegma* is so similar to *Botryllus* and *Botrylloides* that it exhibits the same degree of variation in colour and form of whole colonies. These varieties are even more definite and constant in *Symplegma* than in the Botryllidæ, the commoner

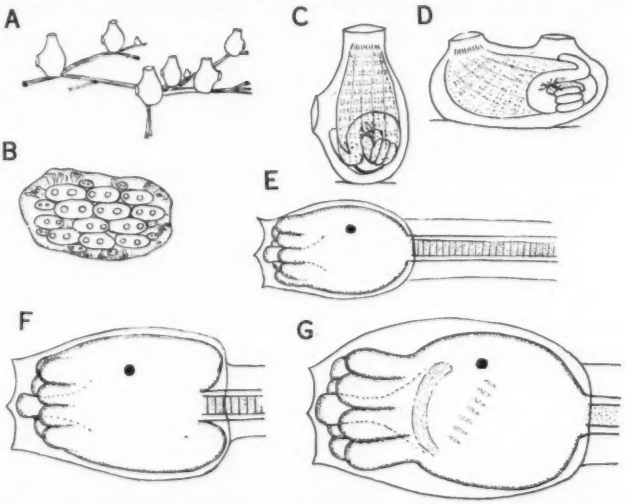

FIG. 5. A, colony of *Symplegma viride stolonica* n.v. B, colony of *Symplegma viride typica*. C, zoöid of *stolonica*. D, zoöid of *typica*. E, F, G, tadpoles of *Symplegma viride*, *Distomus variolosus*, and *Botryllus gigas*, respectively, drawn to the same scale.

colour varieties being,—green, green and white, green and brown, black and white, and orange. With the exception of the last, all colonies are very compact and sheet-like as in botryllids, the adult zoöids being pressed together, young buds appearing in large numbers together with clusters of ampullæ at the colony margin. The orange variety, however, is different. Instead of the budding stolons being very short, they are even longer than in *Stolonica socialis*, with the result that the zoöids are often widely separated from each other while the individual zoöids assume a comparatively vertical position. It may, in view of this difference, be worth recognizing the orange

form as a definite variety. If so, it is suggested that it be named *Symplegma viride stolonica*.

The essential unity of such forms as *Symplegma*, *Botryllus*, and *Distomus* is perhaps best shown in the study of the development.

The tadpole of *Symplegma* is almost identical with those of *Distomus*, *Stolonica*, and *Styelopsis*, except that it is somewhat smaller. It has the same degree of organization; it has the same single but composite sense organ (probably developed from the otolith after the primitive eye was lost, an otolith and eye being found only in *Styela* itself); and in *Symplegma* and *Distomus* there is a long anterior mental process surrounded by a ring of ampullæ. *Botryllus* and *Botrylloides* both produce tadpoles with a similar mental process and ring of ampullæ, there being eight ampullæ in each of these genera and also in *Symplegma*. While the botryllid tadpoles are the more highly organized, the young oozoöids developing from these and from tadpoles of *Symplegma* are hardly to be distinguished.

Altogether there are greater differences between a genus such as *Styela* and the polystyelids than there are between these last and the botryllids.

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THE LIFE HISTORY OF EPIBDELLA MELLENI MACCALLUM 1927, A MONOGENETIC TREMATODE PARASITIC ON MARINE FISHES

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At present our knowledge concerning the life histories of the members of the order Monogena is extremely scanty. Although the life histories of the monogenetic trematodes are apparently much simpler than those of the digenetic forms, they have been less extensively studied, and definite information concerning them is, with a few exceptions, fragmentary. Of the sub-order Monopisthodiscinea Fuhrmann the only life history known is that of *Gyrodactylus* (Katharíner, 1904). Of the sub-order Monopisthocotylinea Odhner, which includes *Epibdella*, no life histories have been reported. In the sub-order Polyopisthocotylinea Odhner two life histories, those of *Polystomum integerrimum* (Zeller, 1872, 1876) and *Diplozoön paradoxum* (Zeller, 1872*a*), have been reported. The dimorphic development of *Polystomum integerrimum* on the gills of the tadpole and in the urinary bladder of the frog as given by Zeller (1872) has been questioned by several workers, including Stunkard (1917) who states that "the findings of Zeller are so unusual that one is led strongly to suspect he confused two different species." This leaves our definite knowledge of monogenetic life histories limited to two genera, *Gyrodactylus* and *Diplozoön*. The present investigation of *Epibdella melleni* MacCallum 1927 presents the third definitely-known life history of the Monogena and the first of the sub-order Monopisthocotylinea.

The adult of *Epibdella melleni* was first described by MacCallum (1927) as parasitic on the Pacific puffer (*Spheroides annulatus*), the spadefish (*Chaetodipterus faber*), and various species of angel fishes (*Angelicthys* and *Pomacanthus*) from the New York Aquarium. Due to the necessarily closed salt water system of the Aquarium, the infection spread rapidly to all tanks which contained susceptible fishes, and for several years it has been a very grave source of danger to all susceptible fishes.

This study was undertaken at the suggestion of Mr. Charles M. Breder of the New York Aquarium and Professor H. W. Stunkard of

New York University. The writers wish to thank Mr. Breder for his constant aid in the collection of material, for the identification of the fishes, and for information regarding their susceptibility, and Professor Stunkard for his helpful suggestions during the preparation of the manuscript. This study was made possible by a grant of the New York Zoölogical Society in the form of a Research Fellowship which was held by the senior author during the summer of 1930 and by the junior author during the academic year 1930–31.

Material and Methods

All material used for this study was obtained from infected fishes at the New York Aquarium. Adult specimens were obtained by scraping the body and cornea of the fish with a scalpel. In this way considerable mucus was obtained along with the parasites. This material was placed in small stender dishes, covered with sea water, and allowed to stand for ten minutes or longer. It was found that the parasites became firmly attached to the bottom of the dish, and that the mucus material could easily be removed with a pipette. After several changes of sea water the organisms, attached to the dish, could be studied with the aid of a binocular. In this way observations of the process of egg-laying were possible. It was found that the organisms remained attached to the dish after fixation. Therefore they were fixed, stained, destained, and cleared while attached by changing the fluid in the stender dish. They were then removed to a slide with the aid of a pipette and covered with balsam. Schaudinn's fluid and saturated aqueous solution of mercuric chloride plus five per cent acetic acid were used as fixatives. Whole mounts were stained with paracarmine, and sections with Delafield's hæmatoxylin and erythrosin.

Larvæ were obtained by collecting eggs and allowing them to hatch. Whenever larvæ were wanted, adult worms were collected and allowed to remain in stender dishes. The worms rarely lived more than twenty-four hours, but during this time numerous eggs were usually laid. These were removed to another dish in which the water was changed several times a day. At the end of five to eight days the eggs hatched, and the larvæ could be isolated with the aid of a mouth-controlled pipette.

Morphology of the Adult

The adult of *Epibdella melleni* was described in some detail by MacCallum (1927), and this original description is substantiated by most of the observations of the present authors. The principal

differences between the description as given by MacCallum and the present material lies in the details of the reproductive system and in the presence of fourteen larval or accessory hooks in the posterior sucker.

The adult of *E. melleni* (Fig. 1) varies from 3.5 to 5 mm. in length. The worms are usually white but sometimes contain numerous small patches of yellow pigment, and the covering of the body is smooth and unarmed. Two pairs of eyes are present near the anterior end of the body. The adhesive organs consist of one large posterior sucker and two smaller anterior suckers, all located on the ventral side of the body. The posterior sucker is round, smooth, and very shallow. It is 1.2 mm. in diameter in 4.5 mm. specimens and bears three pairs of large spines but no papillæ. The most anterior pair of spines is about .22 mm. in length, is slightly forked at the base, and is directed anteriorly, while the other two pairs, more posterior in position, arise posteriorly but are recurved in such a manner as to form veritable hooks whose points are directed anteriorly. The middle pair is the largest and most powerful, measuring about .30 mm. in length; the most posterior pair is much smaller, being only about .12 mm. in length, and its recurved points are relatively fine. There are also fourteen very small (10 μ in length) larval or accessory hooks arranged radially close to the margin of the sucker. These hooks are actually present on the larva and are apparently homologous with the larval hooklets of other monogenetic trematodes. Each of these hooklets is branched, and the two points are opposed (Fig. 13). The margin of the posterior sucker bears a secondary ring of very thin muscular tissue which serves to make the closure of the sucker on the substratum more secure and certain (Fig. 3). The anterior suckers are unarmed and bear no extra peripheral ring of muscular tissue. Both arcuate and dorso-ventral muscle fibers may be seen in sections of the suckers, but the greater portion of the suckers is made up of dorso-ventral fibers.

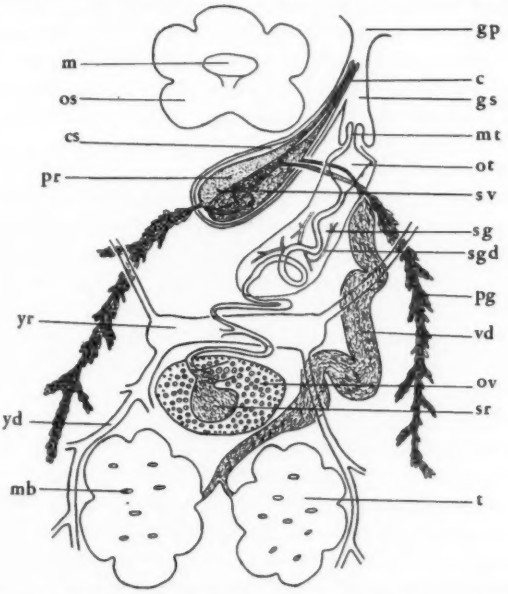
The oral sucker is large and powerful and is located in the median plane behind the anterior pair. The sucker is almost completely covered by thin lip-like structures which are a continuation of the dermal and epithelial layers of the ventral body wall (Fig. 2). These form a flat pouch whose opening coincides with that of the mouth sucker. Apparently these lips must be retracted when the sucker is attached to the substratum. When closed the oral sucker is pentalobate as shown in Fig. 1. It opens directly into the short œsophagus; there is no muscular pharynx. From the œsophagus arise the two main lateral intestinal trunks which have branches from practically all parts of the body. Each lateral intestinal trunk receives about

fourteen smaller branches, and each of these smaller branches is further subdivided. No anastomosis of the branches could be definitely traced in sections, but MacCallum (1927) has described anastomoses of the branches in the median posterior region of the body. However, since anastomosis of the digestive cæca is quite common in other genera of the Monogena, it is possible that it occurs in this species.

The nervous system is composed of a crescent-shaped ganglionic mass anterior to the oral sucker, and two pairs of longitudinal nerve trunks, one of which lies just ventral to the main excretory channels, and the other of which lies more laterad (Fig. 1). The four eyes are embedded in the anterior ganglionic mass. The two trunks on each side are united to each other by three cross-connections, and they fuse in the posterior region of the body so that only two instead of four trunks enter the posterior sucker. In the posterior sucker these two trunks turn mediad and unite, forming a virtual 'ring' system, composed of two pairs of longitudinal trunks united anteriorly by two ganglionic masses and posteriorly by a connection in the posterior sucker. Anteriorly from the anterior ganglionic mass there arise two short trunks which innervate the anterior suckers, and these trunks also are united by a cross-connection. The smaller nerves branch off from the longitudinal trunks and from the cross-connection in the posterior sucker, which seems to serve as a nerve plexus for the numerous small branches which innervate the sucker. In some sections it appeared as if there were another cross-connection, semicircular in shape, in the posterior sucker besides the one shown in the figure, but this could not be traced with certainty.

The excretory pores are two in number and open to the dorsal side of the body, laterad to the posterior margin of the mouth. The two main excretory channels lead posteriorly from the excretory pores and unite near the posterior margin of the body. Slightly posterior to the excretory pores these channels widen to form the excretory vesicles which are very noticeable in the living adult. The longitudinal channels receive many branches, and these branches are repeatedly bifurcated in such a manner that the terminal ends ramify to all regions of the posterior two-thirds of the organism. Anteriorly from each excretory vesicle arises another longitudinal channel which passes forward, is greatly bifurcated, and curves mediad between the oral and anterior suckers. These two anterior channels unite in the median line behind the eyes so that the four main channels form virtually a 'ring' system composed of longitudinal channels united close to the anterior and posterior ends of the worm. The main

channels in the body and also in the posterior sucker are identical in position and in connections with those of the larva which will be described later. The excretory system of the adult is obviously an elaboration of the pattern present in the larva, but the details of the system of the adult have not been traced. It is considered doubtful, however, whether there are four main longitudinal channels posterior to the excretory vesicles as described by MacCallum (1927), and it is



TEXT FIG. 1. Semi-diagrammatic drawing of reproductive system of *Epibdella melleni*. Abbreviations: *c*, cirrus; *cs*, cirrus sac; *gs*, genital sinus; *m*, mouth; *mb*, muscular band through the testis; *mt*, metraterm; *od*, oviduct; *os*, oral sucker; *ot*, oötype; *ov*, ovary; *pg*, prostate gland; *pr*, prostatic reservoir; *sg*, 'shell gland'; *sgd*, 'shell gland' duct; *sr*, seminal receptacle; *sv*, seminal vesicle; *t*, testis, *vd*, vas deferens; *yd*, vitelline duct; *yr*, yolk reservoir.

thought probable that in his description the nervous system has been confused with the excretory.

The male reproductive system (text figure 1) is rather unusual in several respects. There are two testes (approximately .4 by .5 mm. in 4.5 mm. specimens) which lie in the mid-portion of the body; they are almost round in general outline with a lobate margin as seen from the dorsal or ventral surfaces, the amount of lobulation apparently increasing with the size of the specimen. They are not smooth

bodies as described by MacCallum (1927). The testes of adult specimens are pierced by two to ten bands of muscle tissue which extend through the organs in a dorso-ventral direction. Their presence indicates that embryologically the testes might have been formed by the fusion of numerous small testicular bodies. Apparently the contraction of the muscles would force the contents of the testes into the short vasa efferentia which unite to form one very large sinuous vas deferens which passes forward on the left of the ovary. At the level of the expanded portion of the oötype the vas deferens is constricted and turns dorsad and mediad to enter the seminal vesicle. The ejaculatory apparatus consists of a very large pear-shaped cirrus sac located approximately in the median line directly posterior to the mouth; the smaller end of the organ tapers to open into the genital sinus to the left of the mouth. The cirrus sac is divided by a partition into two pyriform sacs: one is the seminal vesicle which receives spermatozoa from the vas deferens entering at the anterior end of the bulbous portion; the other is the prostatic reservoir which receives the prostate secretion. The prostate consists of two long, narrow, slightly branching, acinous glands; the duct of one enters the cirrus sac at its anterior end, and of the other at the anterior end of the bulbous portion at the same level as does the vas deferens. The posterior ends of the seminal vesicle and of the prostatic reservoir are folded back upon themselves in some of the specimens examined. The cirrus is a long, slender, heavy-walled tube continuous with the wall of the cirrus sac. It is divided internally by a septum continuous with its outer wall, thus forming two separate channels which extend to the end of the organ. One of these channels is continuous with the seminal vesicle and the other with the prostatic reservoir. The prostatic secretion stains deeply with erythrosin, and in sections or whole mounts gives a deep red color to the prostate glands, to their ducts, and to the prostatic reservoir. The secretion appears granular in stained sections and hyaline in living specimens. The cirrus is unarmed, but the end is marked off by a slight stricture. The cirrus sac opens into the genital sinus which is divided by a septum into two portions, a medial which receives the cirrus and a lateral into which the metraterm opens.

The female reproductive system of *E. melleni* (text figure 1) is quite simple in outline but has an unusual feature in the position of the seminal receptacle. The ovary lies in the mid-portion of the body, in the median plane, just anterior to the testes. It is oval and measures about .30 by .22 mm. in 4.5 mm. specimens. The seminal receptacle is definitely within the ovary and can be demonstrated as

such in cross, sagittal and frontal sections, thus obviating the possibility that it is above or below the ovary. It may be clearly seen to contain spermatozoa when observed in sections with an oil immersion lens. The oviduct begins near the center of the ovary and is connected close to its origin with the seminal receptacle. Since both spermatozoa and ripe eggs are found in the initial portion of the oviduct and in the anterior part of the seminal receptacle, it is believed that fertilization takes place in this region. The oviduct passes out of the ventro-anterior portion of the ovary and arches dorsad and to the left. It then turns ventrad and crosses under the yolk reservoir from which it receives the yolk duct. The oviduct then continues tortuously to the oötype. The oötype is a long, slender, very muscular, coiled structure with a greatly enlarged tetrahedral anterior end. A uterus is absent, and the oötype opens via the metraterm into the lateral division of the genital sinus. The oötype is surrounded by a rather extensive 'shell gland' embedded in loose connective tissue. The ducts from the 'shell gland' unite to form several common tubules which open into the posterior portion of the oötype. A vagina and genito-intestinal canal are absent, and the vitellaria fill most of the interstitial space between the other organs throughout the body of the worm.

The Process of Egg Laying

In the living adult the yolk reservoir and the vitelline ducts may easily be distinguished with the aid of a low-powered binocular microscope, especially when they are full of yolk cells which appear as large gray structures against the clear body of the organism. When an egg is to be formed, a seemingly large quantity of yolk material streams from the yolk reservoir into the oötype, and it is driven back and forth by a rapid reversible peristaltic movement of this structure. The greater mass of yolk comes to lie in the anterior tetrahedral end of the oötype. During this time the shell material must lie around the yolk, and it is visible as a hyaline covering of the filament of the egg. The filament is at first filled with a central core of yolk material. This yolk material, due to peristaltic movements of the surrounding oötype, is driven forward into the tetrahedral region of the oötype, and further contraction of the posterior region of the oötype results in the shaping of the filament which consists at this stage of only hyaline material. During this time the yolk material at the anterior end of the egg is being shaped into its characteristic tetrahedral form. As the formation of the filament is completed the shell material may be distinguished around the body of the egg. The surging movement of the yolk continues until the egg is com-

pletely shaped, and then the shell material begins to harden. As it hardens it becomes yellowish, and this color gradually becomes deeper. When formation of the egg is complete, the anterior suckers, if attached, release their hold on the substratum, and the anterior end of the organism curves dorsad. The egg is then very quickly shot out from the anteriorly directed genital pore. The filament sometimes remains caught in the genital opening, but more often the egg is thrown completely free of the mother organism. After elimination of the egg, the shell material becomes a very deep yellow-bronze and is quite opaque.

Usually within a few seconds after the discharge of an egg, more yolk material flows from the yolk reservoir into the oötype, and the formation of another egg is begun. The whole process of egg formation requires from two to ten minutes, and the average time is about five minutes. The time-controlling factor seems to be the rapidity with which yolk material may be supplied to the yolk reservoir. Sometimes the yolk reservoir becomes completely emptied, and the process of egg-laying is stopped for a few minutes until the reservoir is refilled from the vitelline ducts. In some few organisms which seemed to lack sufficient shell material, the eggs were incompletely formed. In such cases the shell material usually formed the filament and the posterior portion of the tetrahedron, and the yolk material was shot out in a stream and was followed by the filament at the time the egg would have been released had it been complete (Fig. 12).

The Egg

The egg of *Epibdella melleni* is tetrahedral in form and usually bears a single long filament and two shorter filaments with curved ends which form veritable hooks (Fig. 8). However, a number of eggs have been observed without hooks (Fig. 9), and others have been seen with three hooks and no filament. All of these types of eggs have been observed to come from the same adult, thus eliminating the possibility that they were eggs of different species. The size of the body of the egg is somewhat variable, but usually measures about .13 mm. on the edges of the tetrahedron. The filament is from .8 to 1.2 mm. in length and is sometimes seen to contain small ellipsoidal cavities throughout part of its length. The length of the filament, the presence or absence of and length of hooks, and the number of cavities in the long filament seem to be determined by the amount and distribution of shell material in the oötype at the time the egg is shaped. In some instances slightly larger and somewhat globiform eggs are produced (Fig. 11). These seem to result from a lack of

sufficient shell material or from the presence of too much yolk in the oötype at the time of egg formation. The normal eggs are yellow in color when formed but turn to a golden bronze shortly after they are laid.

Hatching of the Egg, and Behavior of the Larva

Eggs of *Epibdella melleni*, isolated into small stender dishes in which the sea water is changed daily, hatch at room temperature in five to eight days. On the fourth to sixth days the larvæ may be seen within the eggs, and if an egg is broken open the larva is found apparently completely formed but non-motile. Just before hatching, the larva may be seen squirming about within the shell. A small circular opening is formed in the rounded corner of the egg, that is, on the corner which bears neither filament nor hook, but a preformed operculum was not observed. The larva emerges anterior end first. The anterior ciliated portion is thrust out, and the cilia meanwhile beat very rapidly. The larva may remain in this position for fifteen minutes. Then the beating of the cilia slowly pulls the organism out until only the posterior sucker remains within the shell. At the end of another fifteen minutes the larva is usually completely emerged. However, the time required for hatching is presumably affected by numerous undetermined factors. The larva, after emergence, swims very rapidly through the water, pausing only momentarily now and then on the bottom of the dish or on solid objects in the water. Within six hours, if the larva does not become attached to a suitable host, it apparently becomes exhausted, the rate of movement decreases, and the organism finally settles to the bottom, capable of only a slow crawling movement. This is presumably due to a loss of the ciliated epithelium, since such larvæ are always almost completely deciliated. Up to this time the posterior sucker does not appear to be functional.

Morphology of the Larva

The ciliated larva of *Epibdella melleni* (Fig. 4) is approximately 225 microns in length and 60 microns in width. It is slightly flattened dorso-ventrally in the anterior region, and, otherwise, is fusiform in general shape except for a constriction in the region of the mouth. The posterior third of the larva is composed of the posterior sucker which, at the time of hatching and for some time afterward, remains folded in such a manner as to be non-functional as a sucker. The anterior two-thirds of the larva is composed of what becomes the body of the adult, and it is, in general structure, similar to that of the adult except that the digestive and excretory systems are simple and the reproductive systems are not yet developed. The oral sucker is round

and muscular and is located in the anterior part of the middle third of the body proper. It opens into a very short œsophagus which leads into two relatively large digestive cæca. These continue latero-posteriorly almost to the end of the body. No diverticula are present. Anterior to the mouth on the dorsal surface are two pairs of eyes. These are cup-shaped masses of pigment from the cavity of which protrude spherical hyaline lenses. Each of the posterior pair of eyes is approximately 16 microns in diameter and is directed anterio-laterad. Those of anterior pair are 12 microns in diameter and are directed posterio-laterad. The anterior suckers are not distinct sucking disks but are pad-like muscular areas, easily distinguishable in the living form.

The excretory system is composed of two relatively large excretory vesicles which are located slightly posterior and laterad to the mouth, four ducts which lead from them, and ten pairs of flame cells. The vesicles are quite distinct in the living organism and appear as large vacuolar structures. The excretory system opens by dorsal pores located on either side of the mouth as in the adult. The excretory system is shown diagrammatically in Fig. 7. Two large excretory channels extend, one from each vesicle, posteriorly into the posterior sucker. They are joined with each other by a cross-channel at the posterior end of the body proper. Each of these channels gives off an anteriorly directed branch which ends in a single flame cell about halfway between the vesicle and the cross-channel. The longitudinal channels continue into the sucker where each branches and ends in five flame cells as shown in Fig. 7. Leading anteriorly from the excretory vesicles is another pair of channels, one from each vesicle. These unite anterior to the mouth to form one median channel which continues forward between the eyes and branches laterally, and then each branch bifurcates anterio-posteriorly and ends in flame cells. In the region of the mouth are four flame cells, one on each side of the anterio-lateral and posterio-lateral sides of the oral sucker. The channels of these cells are probably branches of the anterior longitudinal channels, but this could not be determined with certainty due to the thickness of the oral sucker and the proximity of the ducts to it. These supposed connections are shown in Fig. 7. The arrangement of the excretory ducts of the larva is virtually a 'ring' system with two lateral excretory pores. The longitudinal channels and cross-connections of the larva have been checked in older specimens and have been found to be the main excretory channels of the adult.

The organism bears cilia in the anterior, middle and posterior regions. The anterior ciliated region extends from the anterior pair

of eyes forward, and cilia cover practically all of the anterior region except the sucking pads. The middle ciliated region, extending from the posterior edge of the excretory vesicles almost to the posterior end of the body proper, is covered with cilia on the lateral, latero-dorsal, and latero-ventral surfaces. No cilia were seen on the mid-dorsal and mid-ventral surfaces in this region. The posterior ciliated region includes the lateral and dorsal surfaces of the posterior two-thirds of the posterior sucker. The cilia are relatively long and arise from an epithelial layer which can be seen distinctly in living specimens. This epithelial layer contains many large highly refractile granules of unknown function. When cilia are lost, the epithelial layer from which they arise is shed with them.

The posterior sucker, as folded when the larva is free-swimming, contains the definitive spines characteristic of the adult. These lie in a longitudinal position with the curved ends directed mediad as seen from the ventral surface. At the margin of the folded sucker, and lying ventrad to the definitive spines, may be seen the accessory or larval hooks. These are all the same size and measure approximately 10 microns in length. A single hooklet is shown in Fig. 13.

When maintained under a sealed coverslip, the ciliated larva may be seen to lose its cilia and to assume a shape and position more characteristic of the adult. The anterior sucking pads are usually very active in fresh preparations; these are stretched forward and are attached to the slide, and the body may be drawn afterward. The organism is capable of moving about in this fashion as well as by the use of the cilia. After this type of movement has been continued for some time, the ciliated epithelium begins to slough off, leaving areas of the normally ciliated region devoid of cilia. Apparently the first regions to become deciliated are those in the vicinity of the anterior sucking pads and those which cover the posterior sucker. However, the other ciliated epithelium is shed shortly afterward. If free-swimming larvæ are selected at random and examined, many are found which do not bear their full quota of cilia due to the sloughing of the epithelium. This may give rise to considerable confusion concerning the normal distribution, and it was necessary to examine a number of specimens in order to obtain the distribution shown in Fig. 4. Concomitantly with the sloughing of the ciliated epithelium, the posterior sucker is unfolded. As it is spread out, the definitive spines turn upon their longitudinal axis so that the curved ends are directed laterad, and the accessory hooks are pointed radially around the margin of the sucker. At this time the posterior sucker is functional and becomes firmly attached to the slide. Such an

organism is shown in Fig. 5. This drawing was made of a larva which was isolated when ciliated and which had undergone the above transformations in about forty-five minutes while under observation.

In an attempt to interpret these transformations in relation to the normal life history of the organism, it is assumed that the larva is free-swimming until it comes in contact with a susceptible fish. Attachment is first by means of the anterior sucking pads. Then the cilia are lost, and the posterior sucker is unfolded, and a firmer attachment is afforded by means of the fourteen accessory hooks of the posterior sucker, aided perhaps by the most posterior pair of definitive spines.

Development of the Attached Form

After attachment the first morphological change to be noticed is the development of the anterior sucking pads into definite suckers. Then the mouth, which is round in the ciliated form, becomes lobate in outline as is that of the adult, and the digestive cæca show signs of becoming diverticulated. The posterior sucker becomes slightly larger in proportion to the size of the body, and the large spines increase in size and change in shape. A specimen in this stage that was obtained from an infested fish is shown in Fig. 6. No increase in size of the larval or accessory hooks could be noted, even when measurements of these hooks in young larvæ and in adult organisms were compared. All accessory hooks measured were between 9.8 μ and 10.7 μ in length. For this reason it is believed that these hooks are not of especial importance in the adult.

Further development of the organism seems to involve principally a further bifurcation of the digestive cæca, an elaboration of the excretory pattern, and the development of the reproductive system. The relatively large amount of space occupied by the reproductive system in the adult is responsible for the great differences in general appearance of the young (Fig. 6) and of the adult forms.

The sizes of the eyes change very little during the growth of the organism. Measurements made of the eyes of newly attached larvæ and of adult specimens showed no significant difference in size. The more anterior pair of eyes was found to be 10–13 μ in diameter, and the posterior pair was 15–18 μ in both young and adult specimens. However, the posterior pair of eyes in the adult is directed anterio-mediad instead of anterio-laterad as in the larva. The eyes of the free-swimming larvæ are quite symmetrical in outline as shown in Fig. 4. Those of the adults are usually not spherical but are flattened in one direction or another. The eyes of some adults even appear conical with the base of the cone adjacent to the lens.

During the growth of the individual the relative amounts of growth of the three pairs of spines are not equal. The middle and the more anterior pairs grow at a rate that is relatively about twice that of the most posterior pair. Thus, the sizes of the first two pairs in the adult are over six times that of the same pairs in the youngest attached form, while the size of the most posterior pair is only three times that of the young form. Table I shows the average measurements of the three pairs of spines in young, medium-sized (about 1.5 mm.), and adult (over 3.5 mm.) specimens, and also the ratio of the sizes of the spines in the various sized specimens, the youngest forms being used as unity. The figures are the averages of the measurements of six specimens, and all three pairs were measured on the same individual. Therefore the ratios are strictly comparable.

Occurrence and Pathogenicity

When the adult of *Epibdella melleni*, obtained from the tanks of the New York Aquarium, was first described by MacCallum (1927),

Table I

	Average size of spines in mm.			Ratio of the size of the spine to that of the young attached form		
Pair of spines	Anterior	Middle	Posterior	Anterior	Middle	Posterior
Young	.035	.045	.038	1.0	1.0	1.0
Medium	.109	.174	.086	3.1	3.9	2.3
Adult	.216	.297	.118	6.2	6.6	3.0

it was stated that the infection was probably introduced by a Pacific puffer (*Spheroides annulatus*) from California. However, it is the belief of Mr. Breder and his associates that there was no Pacific puffer in the Aquarium for some time preceding the discovery of the parasite and that the parasite was originally introduced and is being continually reintroduced with shipments of fishes from Key West and Nassau. Furthermore, the parasite occurs in both the Chicago and Philadelphia Aquariums, neither of which has Pacific fishes. Also, it is known that *E. melleni* will not survive very long exposure to the acid water of New York harbor and that it will multiply rapidly in the neutral tank water of the Aquarium. For many years no attempt was made to control the chemical composition of the water in the tanks of the New York Aquarium, and only after the installation of an efficient means of chemical control did the parasites become numerous, probably due to the very high acidity of the water previous to that time. For these reasons it is believed that the parasite is a West

Indian species and that it might have been continually present in small numbers in the New York Aquarium for many years before its discovery in 1927.

The fishes which have been found to be susceptible to infection with *Epibdella melleni* are as follows:

Subclass Teleostomi
Order Acanthopteri
Family Carangidæ
Caranx crysos (Mitchill), Runner (X)
Caranx hippos (Linnaeus), Common Jack (X)
Naucrates ductor Linnaeus, Pilot Fish
Trachinotus carolinus (Linnaeus), Common Pompano
Trachinotus glaucus (Bloch), Old Wife or Palometa
Vomer setapinnis (Mitchill), Moonfish
Family Pomatomidæ
Pomatomus saltatrix (Linnaeus), Bluefish
Family Serranidæ
Centropristus striatus (Linnaeus), Common Sea Bass (X)
Dermatolepis punctatus Gill, Spotted Grouper (Pacific) (X)
Epinephelus adscensionis (Osbeck), Rock Hind
Epinephelus guttatus (Linnaeus), Red Hind
Epinephelus morio (Cuvier and Valenciennes), Red Grouper (X)
Epinephelus striatus (Bloch), Nassau Grouper (X)
Paralabrax maculatofasciatus (Steindachner), Spotted Cabrilla (Pacific)
Promicrops itaiara (Lichtenstein), Jewfish
Family Lutianidæ
Lutianus analis (Cuvier and Valenciennes), Muttonfish
Lutianus apodus (Walbaum), Schoolmaster
Lutianus jocu (Bloch and Schneider), Dog Snapper
Lutianus synagris (Linnaeus), Spot Snapper (X)
Family Hæmulidæ
Anisotremus surinamensis (Bloch), Black Margate
Anisotremus virginicus (Linnaeus), Porkfish
Hæmulon album Cuvier and Valenciennes, Margate
Family Sciænidæ
Menticirrhus saxatilis (Bloch and Schneider), Kingfish
Micropogon undulatus (Linnaeus), Croaker
Family Labridæ
Lachnolaimus maximus (Walbaum), Hogfish
Tautoga onitis (Linnaeus), Tautog
Family Ephippidæ
Chætodipterus faber (Broussonet), Spadefish
Family Chætodontidæ
Angelichthys ciliaris (Linnaeus), Queen Angelfish
Angelichthys isabelita Jordan and Ritter, Blue Angelfish
Angelichthys townsendi Nichols and Mowbray, Townsend's Angelfish
Chætodon ocellatus Bloch, Common Butterfly Fish
Pomacanthus arcuatus (Linnaeus), Black Angelfish
Pomacanthus paru (Bloch), French Angelfish
Family Acanthuridæ
Acanthurus cæruleus Bloch and Schneider, Blue Tang
Acanthurus hepatus (Linnaeus), Brown Tang or Doctor Fish
Family Balistidæ
Balistes vetula Linnaeus, Queen Triggerfish
Melichthys bispinosus Gilbert, Pacific Black Trigger (X)

Family Monacanthidæ
Ceratacanthus schæpfii (Walbaum), Orange Filefish
Stephanolepis hispidus (Linnaeus), Common Filefish
Family Ostraciidæ
Lactophrys tricornis (Linnaeus), Cowfish
Lactophrys trigonus (Linnaeus), Common Trunkfish
Lactophrys triqueter (Linnaeus), Smooth Trunkfish
Family Tetradontidæ
Spheroides annulatus (Jenyns) Pacific Puffer (X)
Spheroides maculatus (Bloch and Schneider), Common Puffer or Northern Swellfish
Family Diodontidæ
Diodon hystrix Linnaeus, Porcupine Fish
Family Triglidæ
Prionotus evolans (Linnaeus), Striped Sea Robin
Family Malacanthidæ
Malacanthus plumeri (Bloch), Sandfish

The species marked "X" seems to have developed a partial immunity after a short period of susceptibility. Most of these species are usually present in the tanks of the Aquarium, and newly arrived specimens always show a marked susceptibility to infection. However, after being present in the tanks for several weeks these species seldom show a slight and never a serious infection although they are continually exposed to reinfection. Some of the other susceptible fishes (*e.g.*, *Chætodipterus faber*, the spadefish) seemingly retain their infections, continually become reinfected, and die if they do not receive treatment. The central members of the spiny-rayed fishes (Acanthopteri), especially members of the families Serranidæ and Lutianidæ, are extremely susceptible, and the possibility of the development of an immunity seems to be more strongly suggested in these families although it is not shown by all members. The other species showing a distinct susceptibility are rather scattered phylogenetically but are all within this order.

Epibdella melleni has never (prior to June 1931) been observed on any of the following fishes, all of which have been continually exposed to infection while at the Aquarium:

Subclass Elasmobranchii
Order Asterospondyli
Family Ginglymostomidæ
Ginglymostoma cirratum (Bonnaterre), Nurse Shark
Family Galeidæ
Mustelus canis (Mitchill), Smooth Dogfish
Family Carchariidæ
Carcharias littoralis (Mitchill), Sand Shark
Order Batoidei
Family Rajidæ
Raja eglanteria Lacépède, Clear-nosed Skate

Family Dasyatidæ
Dasyatis centrura (Mitchill), Northern Sting Ray
Family Aëtobatidæ
Rhinoptera quadriloba (Le Sueur), Cow-nosed Ray

Subclass Teleostomi
Order Apodes
Family Murænidæ
Gymnothorax funebris Ranzani, Green Moray
Gymnothorax moringa (Cuvier), Spotted Moray
Order Haplomi
Family Pœciliidæ
Fundulus heteroclitus (Linnaeus), Common Killifish
Fundulus majalis (Walbaum), Striped Killifish
Order Lophobranchii
Family Syngnathidæ
Hippocampus hudsonius De Kay, Northern Sea Horse
Order Acanthopteri
Family Serranidæ
Mycteroperca bonaci (Poey), Black Grouper
Mycteroperca microlepis (Goode and Bean), Gag
Roccus lineatus (Bloch), Striped Bass
Family Lutianidæ
Evoplites viridis (Valenciennes), Blue-striped Snapper (Pacific)
Lutianus griseus (Linnaeus), Gray Snapper
Family Hæmulidæ
Hæmulon sp., Grunts
Family Sparidæ
Archosargus pourtalesii (Steindachner), Pacific Salema
Archosargus probatocephalus (Walbaum), Sheepshead
Family Sciænidæ
Leiostomus xanthurus Lacépède, Spot
Pogonias cromis (Linnaeus), Black or Sea Drum
Family Pomacentridæ
Pomacentrus rectifrænum (Gill), Pacific Beau Gregory
Family Diodontidæ
Chilomycterus schœpfii (Walbaum), Spiny Boxfish
Family Echeneidæ
Echeneis naucrates Linnaeus, Shark Sucker
Family Batrachoididæ
Opsanus tau (Linnaeus), Toadfish

It is to be noted that although six elasmobranchs, representing six different families, were exposed, not one was ever observed to be infected. Also, five orders of the teleosts are represented on the list of non-susceptible fishes, and only one order, the Acanthopteri, is represented on the list of susceptible fishes. Therefore it seems as if all susceptible fishes may belong to the order Acanthopteri. Of this order a high susceptibility is shown by members of the families Serranidæ, Lutianidæ, and Ephippidæ, and no infections have been observed in the families Sparidæ, Pomocentridæ, Echeneidæ, and Batrachoididæ. Some of the other families are represented by

members on both the susceptible and supposedly non-susceptible lists, and others are represented on the susceptible list only.

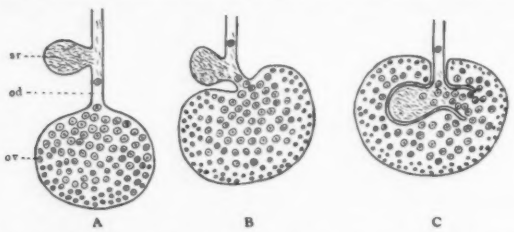
The injury produced to a susceptible fish is usually quite considerable, and if the infection is not treated, death often results. The trematodes attach themselves to the epidermis and to the conjunctiva, and in some cases they have been found in the gill and nasal cavities. Young specimens may be found on almost any part of the epidermis, but in some species (*e.g.*, *Chætodipterus faber* and *Promicrops itaiara*) the adults seem to be concentrated on the eyes. This concentration may be brought about either by the migration of young forms to the eyes, or by a high mortality rate of those which have become attached to the epidermis, or by both of these possible factors. The relative ease with which nourishment could be obtained from the soft tissue of the conjuctiva as compared with the difficulties encountered with the firmer epithelium between the network of scales seems to offer an obvious explanation of why such a concentration might occur. In mild infections the cornea is attacked and sometimes destroyed. If the infection is not treated, destruction of the eye follows, probably due to the effects of both *Epibdella* and secondary bacterial invaders. In very heavy infections the epidermis may suffer such severe injury as to cause the falling off of scales and the exposure of large areas of connective and muscular tissues with subsequent death of the fish. In one case, that of a Galapagos labroid which had escaped attention for some time, over two thousand adult worms were removed from the entire surface of the body. In the case of another similar infection several thousand eggs were found in the gill and nasal cavities. Both of these severe infections resulted in the death of the fishes.

Discussion

The life history of *Epibdella melleni* differs widely from the other definitely-known life histories of the Monogena. The life history of *E. melleni* seems to offer no homologies to the polyembryonic or pædogenetic condition found in *Gyrodactylus* (Kathariner, 1904) or to the characteristic fusion of the diporpa larvæ of *Diplozoön* (Zeller, 1872*a*). The direct development of a ciliated larva into the adult seems to be more closely related to the type of development described for *Polystomum integerrimum* (Zeller, 1872, 1876). However, the dimorphic development described for this species is probably, as pointed out by previous workers (*e.g.*, Stunkard, 1917), the result of the confusion of the life histories of two different species. It seems probable that the ciliated larva described by Zeller may develop into

one of the two forms which he describes as the adult. If the development is similar to that found in *Epibdella*, it is quite probable that the ciliated larva of Zeller developed into the adult which was found on the gills of the tadpole. Also, if the ciliated larva of Zeller is shown to be the larva of the form in the urinary bladder, it is possible that a comparison of the two cases of direct development may still be drawn.

The wide differences between the life histories of *Epibdella* and *Gyrodactylus* seem to have an important bearing on the taxonomic grouping of the members of the order. Fuhrmann (1928) removed from the suborder Monopisthocotylinea Odhner the three families Protogyrodactylidæ, Gyrodactylidæ, and Calceostomidæ and created for them a new suborder, Monopisthodiscinea, of which the distinguishing characteristic was the absence of a definite posterior sucker



TEXT FIG. 2. Diagrams showing the possible development of the intra-ovarian seminal receptacle. A. Relationships of seminal receptacle (*sr*), oviduct (*od*), and ovary (*ov*) in some of the other species of Monogena. B. A hypothetical intermediate stage. C. The relationship as found in *Epibdella melleni*. The boundary between the seminal receptacle and the ovary was not seen as a double wall, but it is quite probable that the two walls fused during development to form one wall as could be seen in sections.

and the possession of a posterior, armed, adhesive disk. A comparison of the life histories of *Gyrodactylus* and *Epibdella*, which may be considered as characteristic members of their respective suborders, seems to offer very good embryological evidence that the separation of *Gyrodactylus* and its close relatives from the other members of the suborder Monopisthocotylinea was well warranted. Thus it seems as if the differences between the two groups are very fundamental and that they may be divided on the basis of their embryology as well as on the basis of their adult morphology.

The presence of the seminal receptacle within the ovary has not, to the knowledge of the authors, been reported previously in the order Trematoda. Inasmuch as a seminal receptacle is found as an evagination of the oviduct in other species of the Monogena, it is thought

that the unusual seminal receptacle noted in *Epibdella melleni* is probably homologous with this structure in other species. Since the oviduct may be traced not only to but also inside of the ovary, it is thought that the intra-ovarian position of the seminal receptacle is brought about either by an overgrowth of the ovary around the seminal receptacle or by the invagination of the receptacle and basal portion of the oviduct within the ovary. Text figure 1 shows how such a rearrangement might have taken place. The wall around the receptacle could not be seen as a double wall as shown in the diagram, but it seems quite probable that the wall of the ovary and the wall of the receptacle might become fused during development, thus giving rise to one relatively thick wall as was seen in sections.

In view of the discrepancies between the original description of *Epibdella melleni* by MacCallum (1927) and the observations of the present authors, it is thought advisable that the description of the species be modified to read as follows:

Family Tristomidæ Taschenberg,
Subfamily Tristominæ Monticelli,
Genus *Epibdella* Blainville 1828,
Epibdella melleni MacCallum 1927.

Ectoparasitic on the eyes and epidermis and sometimes in the gill and nasal cavities of numerous marine fishes of the order Acanthopteri. Body oval, measuring 2–5 × 1.5–3.0 mm., with definite anterior suckers; skin smooth, posterior sucker with two pairs of hooks, one pair of spines, and fourteen very small (10 μ) larval hooklets, but no papillæ. Digestive tract greatly ramified. Cirrus sac pyriform, divided internally to form two saccules, the seminal vesicle and the prostatic reservoir. Two prostate glands; no vagina, uterus, nor genito-intestinal canal. Seminal receptacle within the ovary. Eggs tetrahedral, with two short hooks and one long filament.

Larva approximately 225 microns in length with three ciliated bands in the anterior, middle, and posterior regions of the body. Four large conspicuous eyes. Digestive system simple. Posterior sucker remains closed until after attachment. Development into adult is direct.

Summary

1. The description of the adult of *Epibdella melleni* MacCallum 1927 is corrected and extended, and the complete life history of the parasite is described.

2. The morphology of the larva of *E. melleni*, including the excretory pattern, is described in detail.

3. The general outline of the development of the larva into the adult is traced.

4. Susceptibility to infection with *E. melleni* has been found to be limited to certain families of the order Acanthopteri.

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EXPLANATION OF PLATES

1. Camera lucida drawing of the adult of *Epibdella melleni* showing the principal organ systems. The vitellaria fill most of the interstitial space throughout the body and are not shown in the figure. Size of specimen is 4 millimeters.
2. Sagittal section through anterior half of adult specimen.
3. Sagittal section through the posterior sucker showing the secondary margin.
4. Free-swimming larva of *E. melleni*, drawn from living material, shortly after hatching. Size of specimen, 225 μ.
5. Larva which had been isolated when ciliated and maintained under a sealed coverslip for forty-five minutes, during which time the ciliated epithelium was shed and the posterior sucker opened.
6. Young individual obtained from an infected fish. Size of specimen, 320 μ.
7. Diagram of the excretory pattern of free-swimming larva.
8. Most common type of egg of *E. melleni*. Length of body of egg, 150 μ.
9. Somewhat less common type of egg.
10. Rather unusual form of egg which differs from the others in the ratio of the lengths of the edges of the tetrahedron.
11. Abnormal egg, shape probably the result of insufficient shell material in the oötype at time of formation.
12. Filament as cast out when apparently still less shell material is available for formation of the body of the egg.
13. Larval hook. Specimen 10 μ in length. *a*, edge view; *b*, side view.

Abbreviations

ag anterior nervous ganglion
as anterior sucker
c cirrus
cs cirrus sac
dc digestive cæcum
e eye
ep excretory pore
ev excretory vesicle
gs genital sinus
lh larval hooks
ln longitudinal nerve trunk
m mouth
mb muscular band through testis
mt metraterm
od oviduct
oes œsophagus
ol oral lips
ot oötype
os oral sucker
ov ovary
pg prostate gland
pog post oral ganglion
pr prostatic reservoir
sg 'shell gland'
sgd 'shell gland' duct
sm secondary margin of posterior sucker
sr seminal receptacle
sv seminal vesicle
t testis
vd vas deferens
yd vitelline duct
yr yolk reservoir

PLATE 1

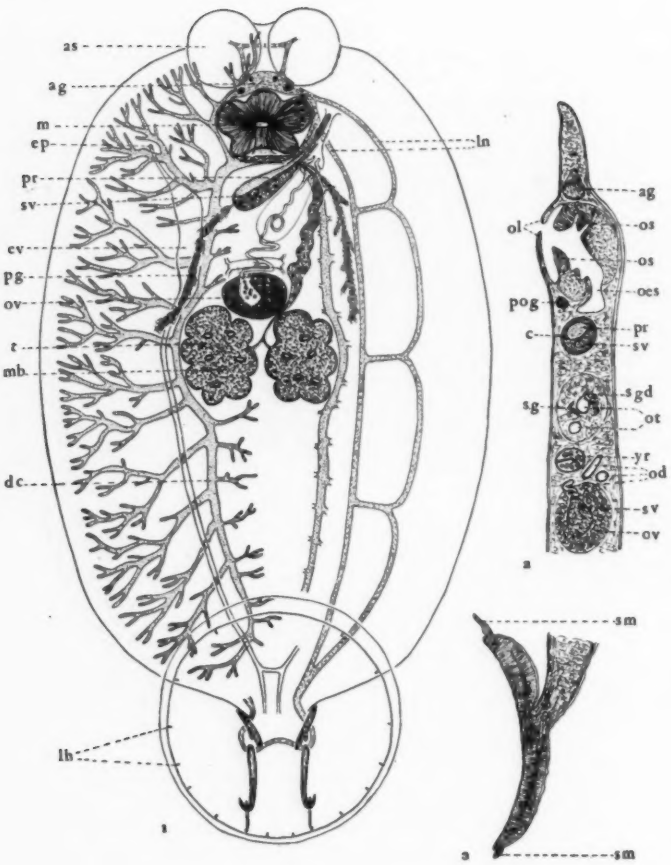
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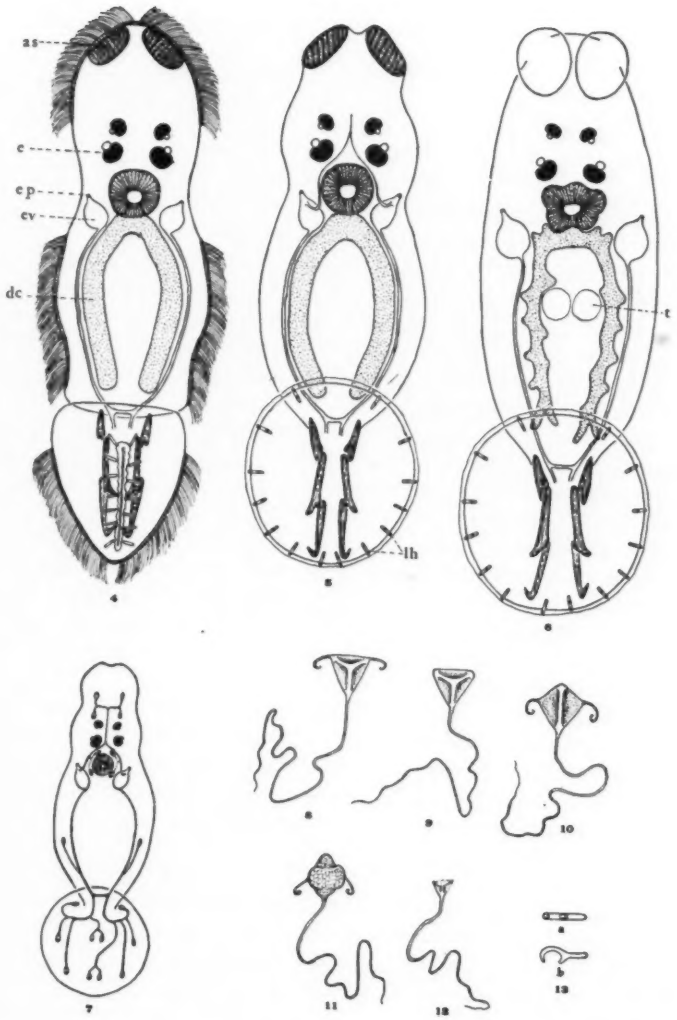
PLATE 2



THE VAPOR TENSION RELATIONS OF FROGS

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Introduction

An account of the water relations of frogs would be incomplete without a description of the exchanges of vapor between the animals and environing atmospheres. Some of the same properties of osmotic pressure and permeability that control water exchanges when frogs are surrounded by liquid might be expected to exhibit themselves in the presence of air. But whereas liquid water may serve as a medium for the exchange of other substances, water vapor is lost from the body without being accompanied by any dissolved material.

The two chief objects of the observations were: first, to find whether the rate of evaporation from the frog's body is proportional to the relative humidity of the atmosphere, or at least fixes a vapor tension curve for the organism; and second, to find whether a frog can come into equilibrium with a definite vapor tension. In theory every object has a measurable vapor tension; in practice the relation to water vapor is greatly complicated by the thermal properties of the body. It therefore becomes necessary to interpret data on rates of evaporation in terms of heat production, body temperature, and thermal conductivity.

Of practical consequence is the finding that under no circumstances can a frog absorb water from the atmosphere. Taken in connection with the fact that the skin offers no unusual obstruction to the loss of water into the atmosphere, it is evident that with respect to water balance a frog is unsuited to non-aquatic existence.

Methods

In the course of the experiments six different procedures were used to establish the relationship between the frog and the atmosphere. Each method was instructive upon certain points and each was useful to evaluate some factors of water exchange. All the methods depended upon weighing the frog at frequent intervals; in these intervals only water was lost in appreciable amounts. The loss of carbon by a frog of 30 grams weight amounts to about 1.3 milligrams per hour, calculating from the mean rate of carbon dioxide production,

measured by Smith (1925) on *Rana pipiens*. The same species was used in all the present measurements of evaporation.

The six methods of weighing evaporation losses that were used will be designated by letters. The last one is to be recommended for most general use in the study of atmospheric relations of organisms.

A. Frogs were exposed to the air of the room in a wire basket resting on a table. The temperature, relative humidity, and dew-point of the air were read at intervals. With inappreciable air motion and small changes of humidity during any one test, the weight changes could be related to the average humidity for the period.

B. A single frog was enclosed in a 400 cc. glass chamber through which conditioned air was recirculated. The chamber was suspended from a triple-beam balance, and connected by flexible rubber tubes with wash-bottles containing sulphuric acid mixtures. The air was pumped by raising and lowering a mercury bulb by means of a "wind-shield wiper"; the wash-bottles, in the bottoms of which were layers of mercury, serving as the valves of the pump. The apparatus was run in a room of constant air temperature, but this did not prevent water from condensing in the chamber when high humidities were used.

C. A single frog was placed in a screen cage in a 4-liter jar equipped with a fan. The fan was spun very rapidly by a belt and motor outside the jar, the fan shaft piercing a brass top fitted by a groove to the jar. The fan was stopped and the jar was opened each time the frog was to be weighed. In the bottom of the jar was a sulphuric acid mixture which controlled the relative humidity of the air. The fan and the air movement produced considerable heat so that temperature gradients always existed. The steady state was temporarily destroyed at each removal of the frog for weighing. The apparatus was operated in the constant temperature room.

D. The fan chamber was used, but the cage containing the frog was suspended from the balance arm by three wires passing through three holes in the chamber lid. The holes were closed by felt washers except when weighings were being made, at which time the fan was also stopped. The chamber was immersed in a regulated water-bath up to the rim, but most of the heat gradients persisted.

E. The frog rested on a screen platform in an ordinary desiccator above a sulphuric acid mixture. The desiccator was immersed in the water-bath up to the rim and was opened to remove the frog at each weighing. In weighing, the frog was exposed to another atmosphere and to handling, and the results were therefore unreliable at slow rates of drying. The air was quiet except as it was moved by the frog's breathing, but the frog remained at a constant distance (5 cm.) from the equilibrating solution.

F. A frog was suspended in a jar by a single wire from a balance arm. The jar and top were completely immersed in a water bath. The wire passed through a vertical tube in entering the jar, and the tube was ordinarily closed by a washer on the wire; when a weighing was taken the wire was raised slightly. The weights were reproducible enough so that an analytical balance was used. The cage proved to be a complicating factor because water coated its wires through capillary attraction away from the frog's skin. The most constant results were obtained by pithing the frog and suspending it by fine wires in a horizontal position. This method was accurately checked by exposing solutions of diverse vapor tensions in a glass dish in place of the frog.

High humidities were obtained by exposing the air to water or salt solutions of known concentrations. Lower humidities were controlled by keeping the air in contact with mixtures of sulphuric acid and water; their concentrations were estimated from their specific gravities measured with a Westphal balance. The vapor

tensions of all these solutions were obtained from chemical handbooks.

Recovery from desiccation was studied in about half of the experiments. The frogs were placed in tap water and weighed at frequent intervals in the manner usual for wet frogs (Adolph, 1931).

Rates of Evaporation

Frogs were gently blotted with a towel before each experiment so that no water would drip from their surfaces. Urine was pressed out of the bladder in the course of handling them, and it is well known that urine formation ceases when water is no longer being taken into the body (Adolph, 1927). Under these circumstances regular changes

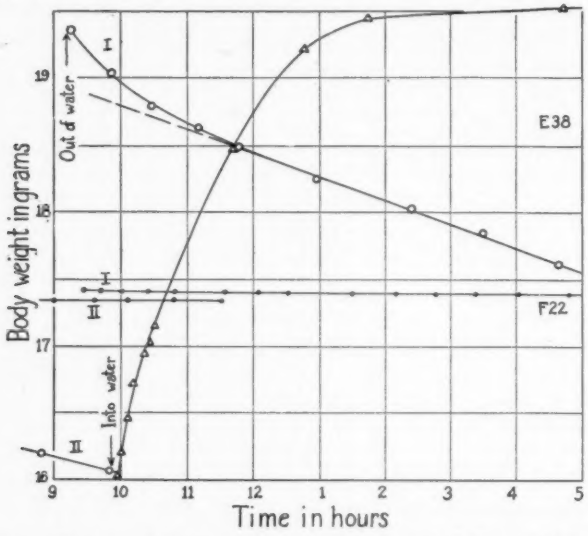

Fig. 1. Changes of body weight during evaporation (by method *E*) and recovery in tap water. The evaporation occurred in an atmosphere saturated with moisture at 20° C. during 25 hours, with slight decrease in rate throughout. For comparison of methods, the rate of evaporation by method *F*, during 26 hours exposure to a saturated atmosphere at 20° C., is also shown. In each case *I* and *II* represent successive days, each experiment being continued through the intervening night.

of body weight were observed. Temperature adjustments all occurred within the first half-hour; then weight was lost rapidly for one or two hours, after which the rate of loss was quite constant from hour to hour. This series of events is shown for one experiment in Fig. 1. Within the next 24 hours the rate of weight loss in any humidity usually decreased very slowly. This change of evaporation rate was

as great as 50 per cent when the atmosphere was saturated, but was less than 20 per cent when the humidity was nearly zero.

The responses of a single individual upon successive exposure to diverse humidities is shown in Fig. 2.

The various experiments were compared by plotting the rates of evaporation against the relative humidities that prevailed. It may be stated that no object is known for whose vapor equilibrium the absolute humidity has significance apart from the relative humidity. This principle follows from the kinetic behavior of gases at uniform temperature. In the experiments it was assumed that the relative humidity was that which would have prevailed if the atmosphere were completely in equilibrium with the equilibrating liquid; this liquid always exposed more surface than the frog. In many experiments a hair-hygrometer was placed in the chamber with the frog and this assumption was found to be nearly true.

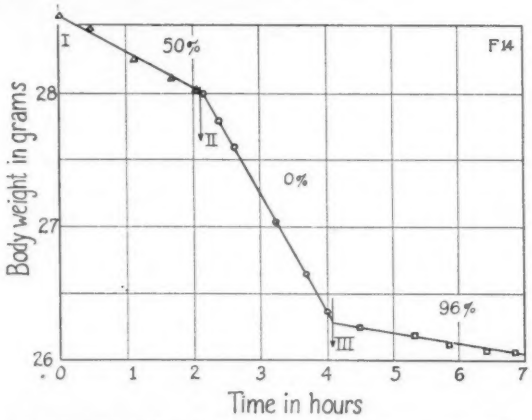

FIG. 2. Changes of body weight during successive exposures (by method *F*) to three relative humidities.

The rates of evaporation may be estimated in a number of ways; by finding the percentage of the original body weight lost in 24 hours, or the average number of grams of water lost per hour in the first six hours, or the grams per square centimeter of body surface lost during the steady state of the third to seventh hours of an experiment. Actually the experimental results were analysed in these several ways, and the last one was adopted, both as giving the most consistent and reproducible data and as being the most rational.

All the results by the six methods are shown as averages in Fig. 3.

Since the rates of evaporation by three different methods fall on curved lines, it is probable that the rates are not exactly proportional to relative humidities. This may be due to some feature of the experiment such as the gradient of vapor near the frog's skin and is not necessarily to be ascribed to the supply of moisture on the surface of the body. One important factor is that, as Hall and Root (1930) observed, the body temperature is much lower than the air temperature as the humidity declines.

A serious attempt was made to relate the rates of evaporation in high vapor tensions to the rates of water exchange by the frog im-

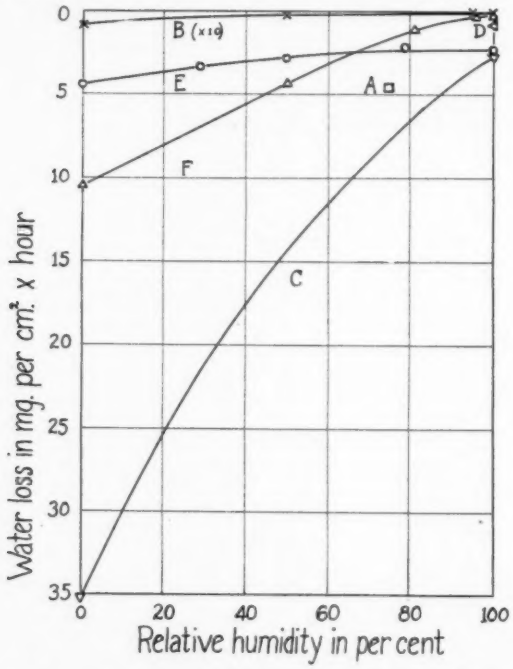

FIG. 3. Mean rates of evaporation at various relative humidities as obtained by the six methods.

mersed in salt solutions of the corresponding tensions. It is well known (Durig, 1901; Adolph, 1925) that in a certain range of sodium chloride solutions that are hypotonic when compared with the body fluids of the frog, water is gained by the body faster than it is gained in tap water. Was it possible that the vapor tension of the frog was higher at 99.8 per cent relative humidity than at 100 per cent? When

compared (by method *C*), no significant difference in rate of evaporation was found at these two humidities. But this merely meant that the precision of the method was too low. In fact none of the methods was nearly good enough to decide this point, because the evaporation was slow even with high velocities of air motion, and because the production of heat by the frog as well as by the air motion could not be sufficiently corrected for.

Vapor Tension Equilibrium

Is it possible to establish conditions in which a frog will neither gain nor lose water? The only procedure sufficiently accurate to answer this question was method *F*. Some 30 experiments were run with saturated atmospheres; the air temperature being constant to $\pm$ 0.01° C., and fluctuations of weight due to all causes being reduced to $\pm$ 0.5 milligram per hour. The average result was a loss of 4.3 milligrams per hour by the frog, or perhaps 3.0 milligrams if the loss of carbon is allowed for. In 5 of the experiments slight gains of weight were shown and in 6 more experiments no change of weight occurred. But in every one of these 11 tests water was later found on the wire cage, and in all experiments where the wire cage was omitted and the pithed frog merely hung in the chamber some weight was lost. A few experiments where the humidity was reduced to 99.7 and 99.3 per cent showed similar losses of weight from the frog.

It is believed that no means could be devised of bringing a living frog into vapor tension equilibrium. The reason for this is, of course, that the organism is producing heat, and that at the frog's surface exists therefore a slight vapor deficit. A frog weighing 30 grams produces 12.5 calories per hour (Smith, 1925), and has a body surface of about 75 sq. cm. (Adolph, 1931); hence in a steady state it is losing 0.17 cal. per sq. cm. per hour. The evaporation from the frog of 4.3 milligrams of water per hour is equivalent to an expenditure of latent heat of 0.034 cal. per sq. cm. per hour. In other words, even this rate of evaporation dissipates only one-fifth of the heat that is being basally produced. Moreover, it eliminates between two and three times the amount of water that is being basally produced in the frog's body by oxidation, which is 1.6 milligrams per hour. The loss of 20 per cent of the frog's metabolic heat by evaporation in this atmosphere happens to be similar to the loss of 24 per cent of a man's metabolic heat by evaporation under basal conditions. It is obvious that no vapor equilibrium can be approached more closely than this by the metabolizing organism.

Effects of Pithing and of Removal of Skin

The best determinations of evaporation rate, as already stated, could be made when the frog was totally quiescent and when the frog could be suspended by fine wires instead of being put into a cage. A few experiments were therefore made to compare the pithed frog with the normal frog. This was best done at high rates of evaporation, because of the smaller importance of absolute errors under such conditions. One experiment is shown in Fig. 4, and it is evident, as was true in other similar experiments, that no consistent difference existed in rates of evaporation between the pithed and the normal frog. A similar conclusion was reported by Hug (1927).

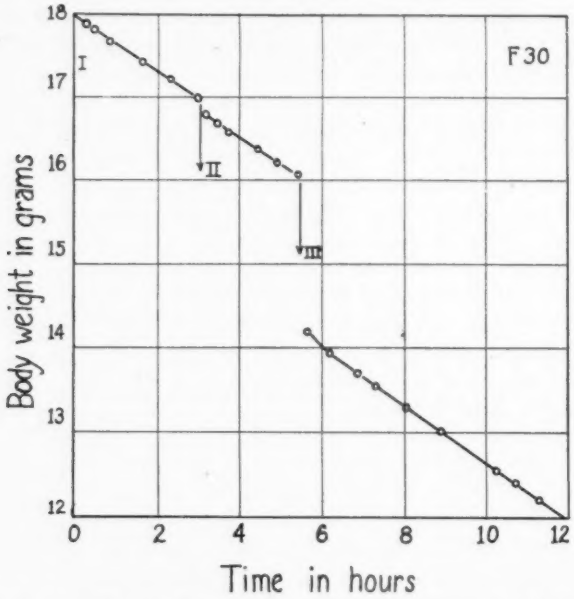

Fig. 4. Changes of body weight during successive exposures of the same frog under three conditions to a relative humidity of 50 per cent. *I*, normal; *II*, pithed; *III*, skinless. Method *F*.

After each experiment it was ascertained that the circulation of the blood persisted in the pithed individual. Two tests in which the circulation was completely stopped showed no detectable difference from the normal frog, and it is likely that the circulation is not a limiting factor in the rate of evaporation. It was concluded by Hug (1927) that dead frogs evaporated at the same rates as living ones.

During and after the evaporation tests it was noted that the appearance and feel of dryness in the skin was highly variable. But it proved impossible to correlate this condition with the rate or the amount of desiccation suffered.

In a short series of experiments the entire skins were removed from pithed frogs. It seemed possible that the external surface of the skin, being in equilibrium with fresh water, would naturally have the vapor tension of pure water, while the deeper tissues would have vapor tensions similar to that of a Ringer's solution, which corresponds to 99.7 per cent relative humidity. It was found that in low humidities the rate of evaporation of a skinless frog was little different from the rate of a normal frog, as Fig. 4 shows. This is in marked contrast to the protection against evaporation furnished by the skins of reptiles (Gray, 1928).

In saturated atmospheres also no difference of rates could be measured. Whereas normal frogs lost 4.3 milligrams per hour, six skinless pithed frogs lost on the average 3.6 milligrams per hour, which is a much better agreement than could be expected. Obviously the heat production of the frog is sufficient to prevent water from condensing on the superficial tissues even though its vapor tension be slightly lower than the tension of pure water.

In a number of experiments frogs were first desiccated by 15 to 35 per cent of their body weights and then placed in saturated atmospheres. In no case was there a significant gain of weight; on the average the rate of loss was the same as for a normal frog. Even when the desiccated frogs were pithed and skinned no gains of weight were found. Evidently the vapor tension of the body cannot by this means be lowered sufficiently to overcome the vaporization due to dissipation of metabolic heat.

Rate of Regain of Water after Drying

But when put into water, a desiccated frog regains fluid at a rapid rate. The course of this regain is shown for one experiment in Fig. 1. The rate is fairly uniform for the first hour or two hours, though some gradual diminution in rate occurs. After the original weight of the frog has been attained, the gain ceases quite sharply.

The average initial rate of gain (38 experiments) was 0.8 gram per hour or 11 milligrams per square centimeter of body surface per hour. This is more rapid than the fastest desiccation in still air can be accomplished. The rate of regain is not correlated with the amount of desiccation, provided at least 5 per cent of the body weight had been lost, nor with the velocity of the desiccation.

Partial contact of the body with moisture is sufficient to supply water for regain (Stirling, 1877; Durig, 1901). If the dried-out frog is merely placed on a damp towel, water will be imbibed through the skin at the average rate. So far as is known to investigators generally, frogs never ingest water through the mouth when immersed in it.

It is of interest that the rate of respiratory metabolism increases with moderate desiccation of the frog and decreases markedly with extreme desiccation (Caldwell, 1925).

Heat Exchanges

It has been demonstrated that the exchanges of water between frog and atmosphere do not correspond to an ordinary vapor equilibrium. The explanation is found in the continual production of heat in the body. In an atmosphere saturated with moisture at

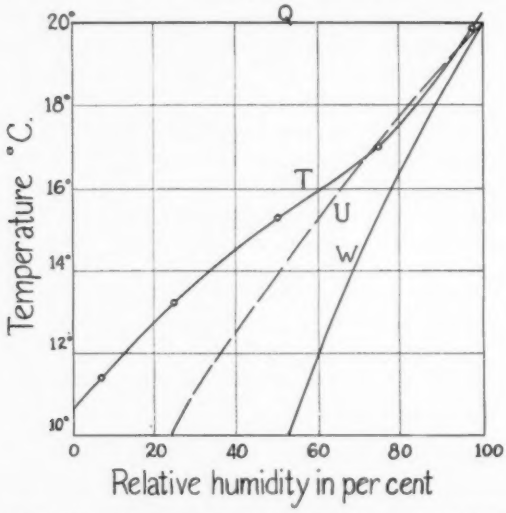

FIG. 5. Humidity and temperature relations at 20° C. *Q*, dry-bulb temperature of the air; *T*, rectal temperature of *Rana pipiens*, data taken from Hall and Root (1930); *U*, wet-bulb temperature of the air; *W*, dew-point temperature of the air.

exactly body temperature this heat cannot be lost by radiation, nor by conduction, nor by convection. Evaporation is also impossible. Hence heat accumulates in the body until the surface temperature rises above that of the surroundings. With each fraction of a degree rise in temperature of the body, more conduction, convection and radiation become possible. The higher temperature now makes possible also evaporation into the warmed layer of air adjacent to the skin.

The body temperatures of frogs in various relative humidities at 20° C. are supplied by the data of Hall and Root (1930); they are replotted in Fig. 5. The vertical distance between the lines *T* and *W* in this figure is the difference of temperature that exists in a steady state between a frog's body and the dew-point of the air surrounding it. This is least at 100 per cent humidity (0.25°), as might be expected.

Comparison with the wet-bulb temperature as obtained with a standard psychrometer (*U*), shows that a frog resembles a wet-bulb

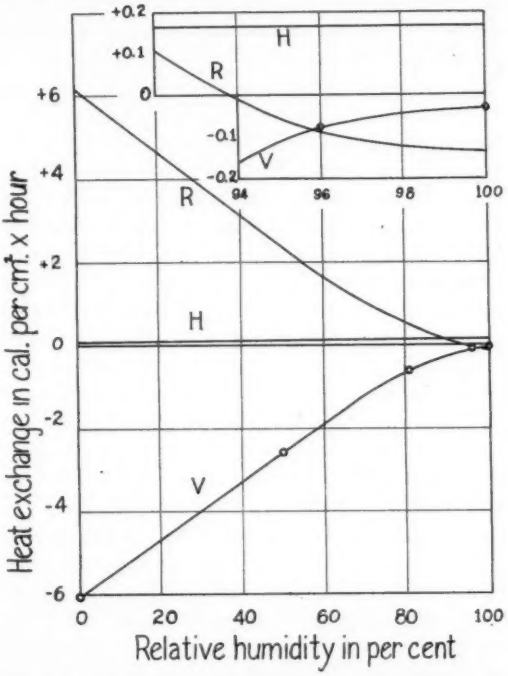

FIG. 6. Partition of virtual heat exchanges by frogs at 20° C. *R*, gain of heat by conduction, convection, and radiation from the surroundings; *H*, gain of heat by oxidative production in the frogs; *V*, loss of heat by evaporation of water from the frog's surface. The inset at the top is a ten-fold enlargement of the right-hand edge of the graph.

thermometer very closely in high humidities. In low humidities the effect of convection in slinging the psychrometer is more pronounced. Hall and Root (1930) had only slight air movement when they measured the rectal temperatures of the frogs.

Evaporation as a complication of measurements of heat production has been discussed, at least for isolated tissues (Fischer, 1927; Hill, 1930). Heat production as a complication of measurements of evaporation deserves equal recognition.

From the rates of evaporation into air of diverse humidities it is now possible to estimate the proportions and total amounts of heat dissipated by vaporization on the one hand, and conduction, convection and radiation on the other hand. It is assumed for this purpose that all the water evaporated from the body gained its latent heat from the frog; this is very nearly true because the specific heat of the body is much higher than that of the air surrounding it. The view is equally sound that the frog really receives no heat from the surroundings while in the steady state, but merely acts as a converter of kinetic heat into latent heat within the atmosphere.

The calculations of heat production are facilitated by the data of Hall and Root (1930) on the body temperatures of frogs in various humidities (Fig. 5), and by the numerous data, as those of Vernon (1897) and Krogh (1914), on the relative rates of respiratory metabolism at various temperatures.

The partition of heat losses from the frog is indicated in Fig. 6, the rates of loss by evaporation being calculated from the measurements by method *F*. It will be seen that under nearly all atmospheric conditions evaporation alone removes heat much faster than combustion generates it. Ordinarily, therefore, the frog is virtually taking up heat from the surroundings by conduction, convection and radiation. It has been ascertained in the present experiments that in 100 per cent humidity, however, the evaporation accounts on the average for only one-fifth of the heat loss. Since the cooling is all produced by evaporation in proportion to the difference of vapor tensions between the frog's surface and the air, it is easily understood why there is no condensation of water on the surface of the frog even though it is much cooler than the atmosphere. The temperature of the frog never decreases to the dew-point of the atmosphere that surrounds it.

It is also possible to calculate roughly a coefficient of heat flow for the combined virtual losses by conduction, convection and radiation, excluding evaporation, from the data of Fig. 6. The heat dissipation (W) is proportional to the body surface (S) to the time (t), and to the temperature difference (θ). Or

$$W = kSt\theta.$$

The best value of k, for the range of low humidities where k is actually

constant, calculated from the slopes of line *R* in Fig. 6 and line *T* in Fig. 5, is 1.0 cal. per square centimeter per hour per degree centigrade. It is more than possible that the curves present in the line for body temperatures (Fig. 5) and in the line for evaporation rates (Fig. 6) are significant, in which case *k* is modified at diverse high relative humidities, and the thermal properties of the frog's body differ at various humidities or body temperatures. Such differences might be due to vasomotor shifts or other physiological responses.

The amount of this heat flow that is due to the single factor of radiation can be calculated. It is assumed that the frog has maximal radiation (as for an ideal black body) such as is believed to hold true for human skin (Cobet, 1924), and that 70 per cent of its surface is exposed to radiation. It is then found that this form of heat transfer might account for half of the combined heat flow (*R*) at low humidities and for all of it at humidities above 70 per cent. Further, it can be calculated from the constant for heat conduction through air that a still atmosphere would be unable to conduct much of the other half of the heat from the water of the bath to the suspended frog. Hence convection currents set up by the frog's breathing and by temperature differences near the body must be important in bringing heat to the animal.

Comment

The failure of frogs to absorb water from moist atmospheres means that these animals cannot survive long away from liquids. While the habits of frogs are such as usually to keep them in or near water, toads are ordinarily regarded as terrestrial. Toads, when subjected to similar vapor tensions, likewise showed no ability to absorb vapor from a saturated atmosphere. Their survival away from water evidently depends upon their taking up water while in contact with wet objects; it has been seen that mere moisture held in towels can supply this. Soil is a sufficient natural source of supply. So far as is now known, a toad has no properties fitting it for water conservation or accretion that are not possessed by most aquatic animals.

The amounts of desiccation endured by frogs have always been matters for remark ever since the first observations were made by Edwards (1824), Chossat (1843) and Kunde (1857). Various investigators have attempted to find how much loss of water is consistent with subsequent recovery; loss of roughly 40 per cent of the body weight, which is 50 per cent of absolute water content, allows of survival (Snyder, 1908; Hall, 1922; Smith and Jackson, 1931).

Studies have been made of the relative losses by the various organs and tissues of the frog's body during desiccation (Durig, 1901;

Ueki, 1924; Iizuka, 1926; Smith and Jackson, 1931). At present little relation can be deciphered between the partition of water losses and the water economy of the body as a whole. It is possible that the marked loss of water by the skin helps to diminish the rate of subsequent evaporation to the small extent found above.

Summary

1. Frogs lose water by evaporation at rates that are nearly inversely proportional to the relative humidities of atmospheres.

2. Functioning of the central nervous system, of the blood's circulation, and of the skin made no significant differences in rates of evaporation.

3. In saturated atmospheres evaporation still goes on, which is explained by the fact that the production of heat keeps the body slightly warmer than the atmosphere.

4. In unsaturated atmospheres heat may be regarded as being lost by evaporation until the lowered temperature of the body comes into a steady state with the gain of heat by conduction, convection, and radiation from the surroundings.

5. No equilibrium of zero evaporation can be established for the living frog, and so the vapor tension of the frog's surface cannot be measured.

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A COMPARISON OF THE PLASTID WITH THE GOLGI ZONE

T. ELLIOT WEIER[1]

There has always been a desire on the part of cytologists to homologize the structural elements of plant and animal cytoplasm. Of late much attention has been given to finding an element in the plant cell which might be comparable with the Golgi zone of the animal cell. Guilliermond (1929), because of the network structure assumed under certain conditions by the plant vacuoles, believed that these might be compared with the Golgi body. This idea found support in the work of Parat (1928), which seemed to show that the Golgi apparatus was mainly vacuolar in nature. Hall (1931), in a recent work on *Euglena*, maintains that it is not the sap vacuole which is comparable to the Golgi body but certain small granules staining with neutral red. Bowen (1920, 1922*a*, *b*, *c*), in an extensive study of insect spermatogenesis, has given a very definite criterion for recognizing the Golgi zone. It is that differentiated mass of cytoplasm which during spermatogenesis gives rise to the acrosome. Working with this in mind, he (1927) undertook a study of spermatogenesis in the moss, *Polytrichum juniperinum*. Unfortunately this investigation was never completed, but Bowen's belief was that the limosphere, a body which because of its development as described by Allen (1917) was thought by Wilson (1925) to be comparable to the acroblast, was elaborated by the osmiophilic platelets. Bowen's results, though differing slightly from the known formation of the acrosome in insects, made it seem possible that the osmiophilic platelets might be compared with the Golgi body.

The problem was attacked independently and in the same manner by Weier (1931, 1932) who, however, arrived at conclusions quite different from those of Bowen. During sporogenesis and spermatogenesis in *Polytrichum commune* reactions of the plastid to osmium and silver techniques and a strikingly comparable structure and behavior of the plastid to that presented by the Golgi body during comparable periods of development led this author to believe that the plastid and the Golgi zone had certain characteristics in common.

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This idea was not new, Bowen having suggested it in 1926 to withdraw it later in favor of the osmiophilic platelet hypothesis.

A study of spermatogenesis in *Polytrichum commune* and *Catharinaea undulata* furnished clear evidence that the limosphere is a derivative of the plastid. Indeed the history of plastid development during spermatogenesis is, in all important details, strikingly similar to the history of the development of the Golgi body during spermatogenesis in insects. When one adds to this the similarity in structure of the living Golgi zone (Morelle, 1926) with the living plastid (Zirkle, 1926), the similarity in reactions of both structures to certain fixing fluids (Morelle, 1926 and Weier, 1932), the similarity of function (the elaboration of specialized products for use by the organism), the similarity of relation to mitochondria in certain cells (Pensa, 1925; Parat, 1928), and the similarity in relation to chemically allied pigments (chlorophyll and hemoglobin—see Weier, 1932), it seems that there must be some significant relation existing between the plastid and the Golgi zone. Furthermore, it has been shown (Weier, 1932) that the previously supposed homology of mitochondria with plastid and of Golgi zone with vacuoles was not sound.

It is well known that osmium and silver impregnation techniques give preparations with a great variety of fixation pictures. Parat in his "vacuome" hypothesis has variously interpreted these to be "vacuoles," "active mitochondria," and "diffuse lipoids." During the course of the sporogenesis and spermatogenesis studies on *Polytrichum commune* and *Catharinaea* fixation pictures surprisingly similar to those interpreted by Parat as "vacuole" and "active mitochondria" were encountered in the plastid. This paper is a record of these fixation pictures. It is believed that the similarity found between Parat's "zone de Golgi" and the plastid is significant in two ways. Firstly, it further establishes the theory that the plastid is comparable to the region of cytoplasm which the zoölogists have generally called the Golgi apparatus. Secondly, it lends strong support to the interpretation of the Golgi body expressed in the work of Bowen, Nassonov, Gatenby, Hirschler, Beams, Morelle and others, namely, that the Golgi body or zone is composed of specialized living cytoplasm and not of a liquid vacuolar sap of a non-living nature as Parat holds.

Figure 1, *A* and *B*, is redrawn from a portion of Parat's (1928) Fig. IX, *a* and *b*. Both *A* and *B* represent, according to Parat, different fixation pictures of the "système vacuolaire" of the salivary gland of the *Chironomus* larva. In *A* fixation has been excellent; the "vacuoles" have impregnated evenly, presenting the aspect which one observes them to have in the living condition. In *B*, however,

"l'élément apparaît incontestablement rétracté. . . . Les aspects les plus divers des vésicules chromophobes à parois chromophiles sont observables. . . ."

The plastid in the living cell of *Polytrichum* is formed of specialized cytoplasm sharply delimited from the remainder of the cell and, if it is a chloroplast, in some manner impregnated with chlorophyll. This

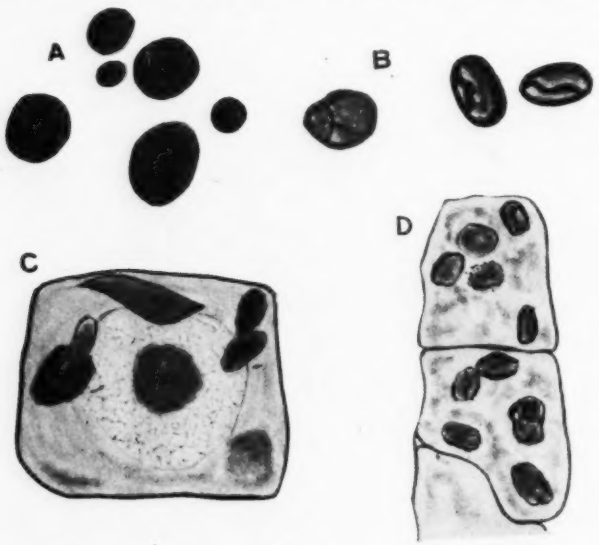

FIG. 1.

A and *B*. Two fragments of the salivary gland of the *Chironomus* larva after fixation according to Da Fano.

A. Cytoplasm insufficiently fixed: the impregnated vacuoles resemble dictyosomes. After Parat.

B. Cytoplasm well fixed: the impregnated vacuoles appear as they do in the living cell. After Parat.

C and *D*. Two aspects of plastid fixation in *Polytrichum commune*.

C. Cytoplasm well fixed: the plastid presents a homogeneous structure similar to that observed in living cells.

D. Cytoplasm poorly fixed: the structure of the plastid has changed so that it may appear similar to dictyosomes or it may have a simple plate-work transversing it.

specialized mass of cytoplasm appears quite homogeneous. Small starch grains may be observed within it after staining with iodine. Osmic acid impregnation upon occasion fixes and stains the plastid so that it appears perfectly homogeneous (Fig. 1, *C*). On the other hand, osmium methods, as well as other techniques, usually do not fix the plastid so perfectly. It most frequently appears in rather

diverse forms, commonly as a chromophobic mass of specialized cytoplasm with the border heavily stained, and a system of one or more plates running through the chromophobic mass, as in Fig. 1, *D*.

It seems then that the plastid and the "vacuole" both appear in the living state as more or less homogeneous masses. Fixation may upon occasion preserve this vital condition (Fig. 1, *A* and *C*). More frequently, however, fixation distorts plastid and "vacuole." Surprisingly enough the distortion is similar in both cases (Fig. 1, *B* and *D*), a "vesicule chromophobe à parois chromophile" or for the plastid a chromophobic mass of cytoplasm with a heavily staining border.

Parat reports a second illustration of poor and perfect fixation of the "vacuoles" in the oöcyte of *Helix*. In the living condition and after mitochondrial fixation the "vacuoles" appear as clear chromophobic regions in the ground cytoplasm of the cell. "Lépidosomes" together with some ordinary mitochondria gather around these areas. These two types of mitochondria are somewhat difficult to separate. In late stages of yolk formation the large "lépidosomes" come into a very intimate relationship with the "vacuoles." After silver impregnation the "vacuoles" contract, and in reducing the silver more heavily on one side come to appear strikingly like the dictyosomes of the classical Golgi body.

In the ovary of *Tulipa gesneriana* Pensa (1925, Figs. 19 and 20) describes an analogous behavior of the young plastids and mitochondria. After fixation according to Meves the young plastids appear as chromophobic regions similar to those which Parat, in *Helix*, has interpreted to be "vacuoles." Mitochondria are grouped around the plastids much as the "lépidosomes" of the *Helix* oöcyte gather around the "vacuoles." After silver impregnation the vacuole-like plastids of *Tulipa gessneriana* either form a small crescent-shaped network with only one side blackened by the silver or appear very much like the classical dictyosomes. That the regions of cytoplasm with which Pensa is working are plastids is perfectly clear from the presence within them of chlorophyll and starch.

Perhaps one of the most striking comparisons between the Golgi zone and the plastid is exhibited in Fig. 2, *A* and *B*. The Golgi zone in *A* is from Marg. and M. Parat (1930, Fig. III, *a*). It illustrates the appearance of the Golgi zone in the pelvic gland of *Triton marbre* after four hours preliminary fixation in the solution of Helly, followed by seventy-two hours post-chromatization at 38° C. This treatment mordants the Golgi zone so that it appears as a darkly-staining mass somewhat hollowed out by "vacuoles" in which the secretory grains first appear.

The plastid of *Zea mays* after identical treatment shows a very similar structure (Fig. 2, *B*). The specialized cytoplasmic mass is easily stained and is found to be furnished with "cavities." Under polarized light the black cross of starch showing within these "cavities" indicates that like the "vacuoles" of the Golgi zone they serve as

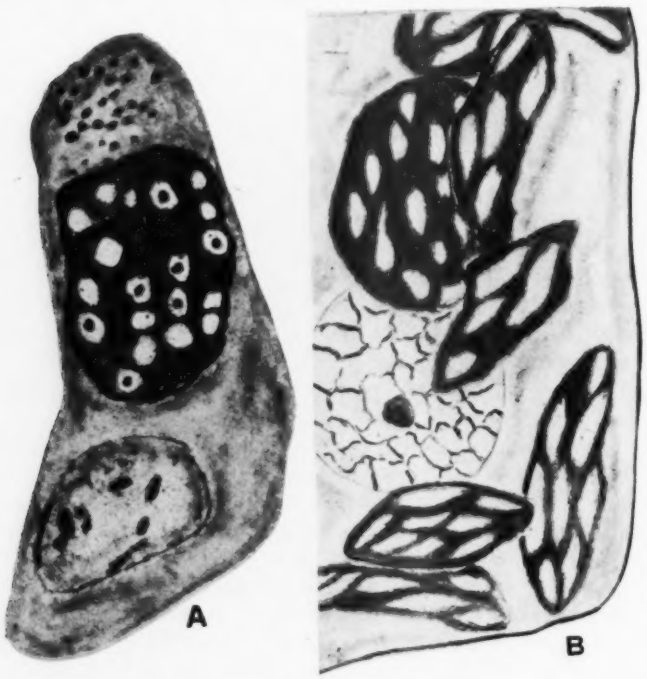

FIG. 2.

A. A cell from the pelvic gland of *Triton marbre* after a post-chromatization of 72 hours. The Golgi zone is a darkly-staining mass containing vacuoles, in which the secretion appears. Active mitochondria are also present. After Marg. and M. Parat.

B. A section of a cell from a *Zea mays* leaf after a post-chromatization of 72 hours. The plastid appears as a densely staining mass containing "vacuoles" in which polarized light demonstrates the presence of starch.

depositories for the secretory products of the specialized mass of cytoplasm.

There is a difference, however, between Parat's description of the Golgi zone and the plastid: the presence of "active mitochondria" in the Golgi zone. According to Parat, a post-chromatization of six

hours is not sufficient mordanting to make possible differential staining of the "active mitochondria." They do, however, show after treatment with osmium and silver. Parat describes these "active mitochondria" as being long filaments. Since they appear only after prolonged fixation and in both longitudinal and oblique sections (Figs. III *a*, IV and V, Marg. and M. Parat, 1930) or in longitudinal and cross sections (Figs. X, XVII *b*, *c*, *f*, *g*, Parat, 1928) as filaments

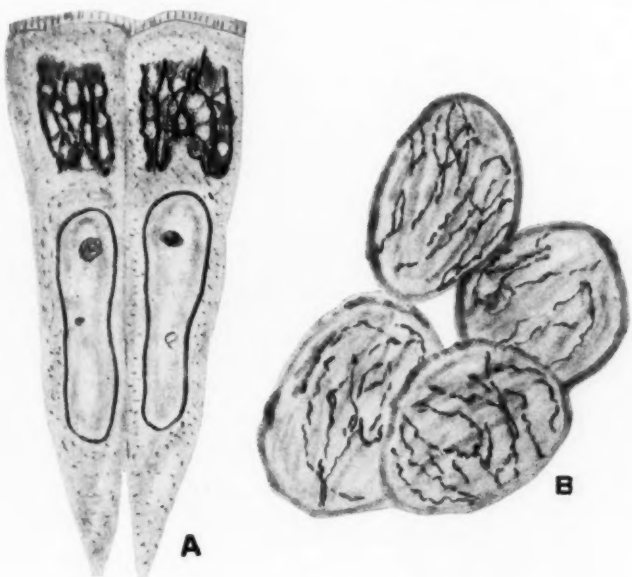

FIG. 3.

A. Silver impregnation of an intestinal cell of *Triton marbre*. Active mitochondria ramify through the diffuse lipoids of the Golgi zone. The vacuoles are frequently in contact with the active mitochondria. After Marg. and M. Parat.

B. Plastid of *Polytrichum commune* after osmium impregnation. Structures similar to the active mitochondria ramify through a denser region of cytoplasm and frequently border vacuole-like regions.

and seldom as granules, is it not possible that these "active mitochondria" are in reality distinct plates such as Morelle and Bowen have described in the Golgi zone?

The plastid in *Zea mays* is evidently more resistant to the action of fixing fluids, for even after seventy-two hours post-chromatization it does not show any evidence of the platework structure which fixation usually imparts to the plastid as well as to the Golgi zone.

Figure 3, *A* and *B*, furnishes another striking comparison between plastid and Golgi zone. In this case the example of Golgi zone structure is taken from Plate XIV, *d* of Marg. and M. Parat, 1930. It represents a silver impregnation of an intestinal cell of *Triton marbre*. Figure 3, *B*, is an osmium impregnation of moss plastids.

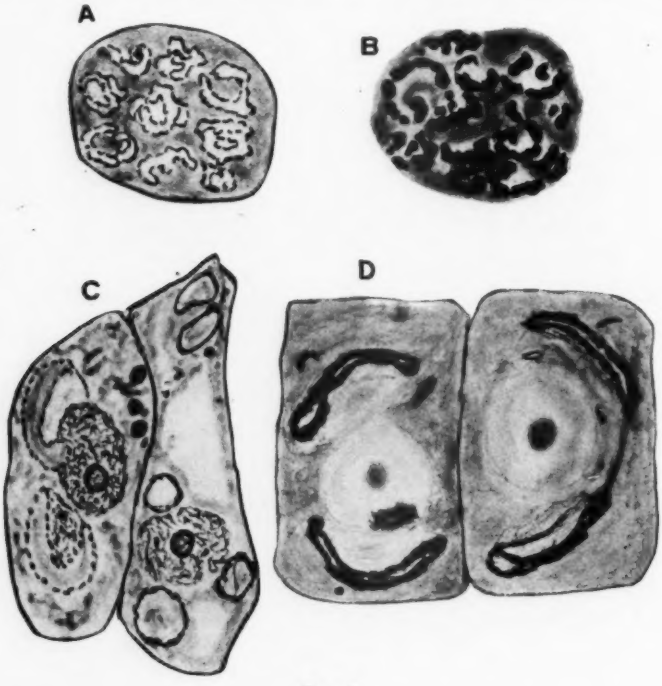

FIG. 4.

A and *B*. Epididymis cells of the mouse; osmium impregnation.

A. Transverse section. Perivacuolar chondriome impregnated. After Parat.

B. Heavy blackened ribbons, formed by the alteration of the perivacuolar chondriome, surround the vacuole, which is deformed but not impregnated. After Parat.

C and *D*. Types of osmic acid impregnation similar to *A* and *B* may be found to occur in the plastid of *Polytrichum commune*.

It will be noticed that in each case the "active mitochondria" lie quite parallel to each other and are most certainly in the case of the plastid and in all probability in *Triton marbre* delicate irregular plates. These ramify through a region of "diffuse lipoids" or specialized cytoplasm which has some power of reducing the osmium, thus

appearing faintly gray. The "vacuoles" of the Golgi zone are clear and round, while the "cavities" of the plastid are longer and not so sharply and clearly delimited from the darker cytoplasm.

In Figs. 2 and 3 we have considered what apparently are good fixations of the "active chondriome" and "vacuome." However, after certain techniques, particularly osmium impregnation, this "active chondriome" may be greatly altered. Figure 4, *A* (Parat, 1928, Fig. XXVIII, *a*) is a cross section of an epididymis cell of the mouse. Here the "chondriome" is well preserved. By making a comparison with the longitudinal section shown in Fig. 3, *A* it may be seen that the "chondriosomes" are most frequently much more ribbon-shaped or plate-like than filamentous. They border on the "vacuoles" which have not blackened with the osmium. In Fig. 4, *B* (Parat, 1928, Fig. XXVIII, *b*) the "active chondriome" has been altered so as to form coarse black plates bordering the "vacuoles" which have not impregnated.

Under a similar treatment with osmium the archesporial plastid of *Polytrichum commune* shows a corresponding reaction. In Fig. 4, *C* an "active chondriome" or filamentous strands of what appears to be plastid cytoplasm border upon a non-blackened portion of the plastid. In Fig. 4, *D* heavy black plates are in contact with the region of the plastid cytoplasm which reduced no osmium.

Both plastid and Golgi zone, then, may under certain conditions precipitate osmium in the form of more or less heavy filaments or plates. These filaments or plates border on a non-reducing mass of plastid or Golgi substance. A comparison of the living plastid with this fixation picture shows that the reduction of the osmium is mainly confined to the borders of the plastonema of the archesporial plastid and that the plastosome, which is certainly of cytoplasmic nature, does not reduce the osmium. It appears lighter than the surrounding cytoplasm, or as a "vacuole." This comparison shows further that the structure of the plastid is not faithfully preserved in that the reduction of the osmium occurs most frequently at the interfaces of the plastonema, the plastosome and the surrounding cytoplasm. This suggests that the blackened regions, instead of marking formed cytoplasmic bodies, may rather indicate interfaces between certain specialized cytoplasmic regions where great cellular activity is taking place. The difference in amount of osmium reduced would depend upon either the length of time the tissue was treated with osmic acid or upon the amount of metabolic activity going on within the cell at the time of fixation. Since it seems that in the plastid this type of impregnation is an artifact in the sense that it does not preserve the exact details of the physical structure of the living cell, may it be

suggested that perhaps a similar phenomenon has taken place in the Golgi zone? In other words, the "active mitochondria" are not mitochondria, permanent cellular elements, but mark instead, regions of intense cellular activity. This activity, as would be expected, is taking place at interfaces between differentiated regions of the cyto-

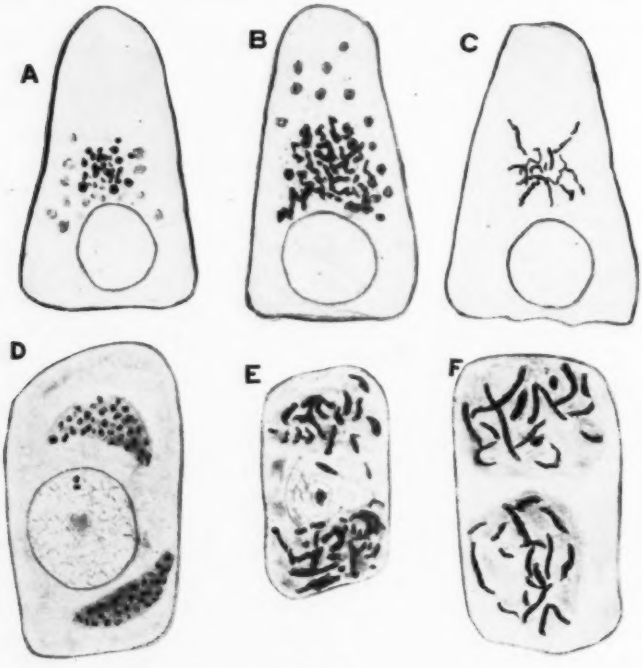

FIG. 5.

A, *B*, and *C*. Metallic impregnation of the pancreas cell of *Scyllium catulus*. *A* and *B*. The vacuome alone has impregnated. After Parat.
C. In this case only the active chondriome has impregnated. After Parat.
D, *E*, and *F*. Osmic acid impregnation of the androgonal plastid of *Polytrichum commune*. The same types of impregnation are encountered here as in *A*, *B*, and *C*.

plasm, either between the Golgi zone and the surrounding cytoplasm or between differentiated regions within the zone.

It is, of course, known that mitochondria do invade the Golgi region (Morelle, 1926, Parat, 1928) but the "active mitochondria" are so different from the ordinary mitochondria in structure and reaction to fixation reagents and so similar to the artifacts due to

fixation and osmification within the Golgi zone (Morelle) and within the plastid that one cannot avoid questioning the interpretation given to these structures by Parat.

In Fig. 5, *A*, *B* and *C* three different aspects of metallic impregnation of pancreatic cells have been reproduced from Parat's (1928) Fig. XXVI. In *A* and *B* only the "vacuome" has impregnated, while in *C* the "active mitochondria" alone reduced the osmium. The criterion for separating these two elements lies merely in the length and the thickness of the bodies which are able to be oxidized by the osmium. Corresponding types of osmium impregnation are frequently encountered in the plastids of androgones within the same antheridium (Fig. 5, *D*, *E* and *F*). In the case of the plastid, one is dealing with

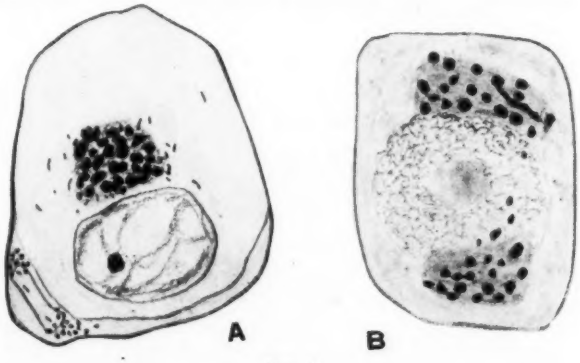

FIG. 6.

A. Nervous cell of the tadpole of *Bufo vulgaris*. Silver impregnation. Vacuome is impregnated as well as a portion of the active chondriome. In the satellite cell the vacuome is impregnated and exhibits a definite polarity. After Parat.

B. Androgonal cell of *Polytrichum commune*. Osmic acid impregnation. Vacuole-like regions impregnate within the plastid.

neither "vacuoles" nor "active mitochondria" but probably with especially active regions of plastid cytoplasm.

Even the Golgi bodies of nerve cells are not without a fixation picture which may be compared to the plastid. Silver treatment on this type of tissue in *Bufo vulgaris* results in an impregnation of the "vacuoles" and "active mitochondria" embedded in a mass of cytoplasm which is more dense than that of the remainder of the cell (Fig. 6, *A*, redrawn from Parat, 1928, Fig. XIV, *b*). At the lower left-hand corner of the large cell is a small satellite cell with its "vacuome" impregnated and showing a definite polarity strikingly similar to that shown by the plastids when there are but two per cell (Fig. 6, *B*).

The plastid of *Polytrichum commune* (Fig. 6, *B*) after treatment with osmic acid not infrequently presents an image quite similar to that assumed by the Golgi zone in the nerve cell of Fig. 6, *A*. Many small regions reduce the osmium intensively so that the plastid appears to have a very similar structure to the Golgi zone of *Bufo vulgaris*. However, the regions in the plastid which reduce the osmium so strongly are apparently not the "cavities" which previously have been compared to Parat's "vacuoles" and within which the starch first appears. They probably represent regions of cellular activity rather than any definite morphological cellular element. The two plastids possess a definite polarity similar to that shown by the "vacuoles" in the satellite cell of Fig. 6, *A*. May it not be that the "vacuoles" too are regions of active cytoplasm rather than

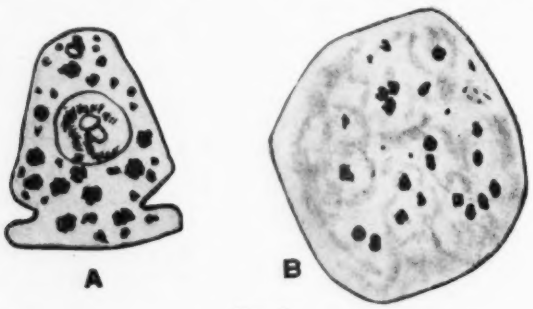

FIG. 7.

A. Salivary gland of the *Chironomus* larva. Vital. The vacuoles appear to be in the form of a network. After Parat.

B. Proplastids in microsporocyte of *Zea mays*. Benda. The proplastids are reticular.

cellular elements perpetuating themselves only by division as the nucleus does?

Parat's classical example of vacuole formation is the salivary gland of the *Chironomus* larva (Parat, 1928, Fig. V, 1 to 8). In the account of this development there are present in all eight stages small discrete "vacuoles." At stage six, however, some of these flow together, forming the secretion. It seems that Parat may have confused the secretory products with the discrete Golgi elements, the region of cytoplasm which elaborated the products.

At any rate young plastids frequently may be observed to have network aspect very similar to Parat's "vacuoles" found in the salivary gland of the *Chironomus* larva. Pensa has reported young plastids of this same structure as occurring in the cells of the young

ovary of *Tulipa gessneria*. In Fig. 7, *B* young plastids found in *Zea mays* microsporocytes are seen to compare very favorably with the "vacuoles" of the salivary gland of the *Chironomus* larva (Fig. 7, *A*).

During spermatogenesis the Golgi zone is formed, according to Parat, of "lépidosomes" or specialized mitochondria, "rhagiocrine and plasmocrine vacuoles," and "diffuse lipoids." The pro-acrosomic granules are associated with the "rhagiocrine vacuoles." Here the "lépidosomes," rather than the vacuoles, as in yolk formation, take on the form of the dictyosome. If we compare the dictyosome-like "lépidosome" (Fig. 8, *A*) with the archesporial and androgonal plastids (Fig. 8, *B*), we find no appreciable difference in structure; both are formed of a convex chromophilic border and an internal chromophobic substance.

FIG. 8.

A. Idiosome from the spermatocyte of *Helix pomatia*. Osmic acid impregnation. The lépidosomes with convex chromophilic and internal chromophobic portions resemble the classical dictyosomes. After Parat.

B. Archesporial plastid from *Polytrichum commune*. Benda or osmium fixation. The plastid closely resembles the dictyosomes.

This might be taken as an indication that the plastid, too, is a specialized sort of chondriosome comparable to the "lépidosome." However, in view of the previous comparison between the plastid and the Golgi zone and the part played by the plastid in the elaboration of the limosphere, this is hardly possible. The rôle of the plastid in the latter respect is identical with that of the classical Golgi body, it elaborates the apical body or acrosome. Parat claims that the "lépidisomes" do not enter into the elaboration of the acrosome. He, however, stresses the fact that they are in an intimate relation with the Golgi zone up until the deposition of the acrosome. Exactly at this time they move away from the zone and pass down the tail of the maturing sperm. It seems that Parat may have missed one of the most important steps in spermatogenesis, the elaboration of the acrosome by the acroblast.

It does not seem possible that the "lépidosome," which is so strikingly similar in behavior and structure to the dictyosomes and androgonal plastid, does not play some rôle in the formation of the acrosome. Nor does it seem possible that the excellent preparations of the late Professor Bowen which the present author has had the privilege of examining can all be either special cases or all distorted to show a relationship between acroblast and acrosome which does not exist in life.

It seems that the similarity shown by the plastid to the Golgi zone cannot be without some significance. It must mean that these two regions of the cell are comparable, possibly even homologous, although the great evolutionary distance between the mosses and animal forms under consideration makes such a suggestion extremely hypothetical.

Since we know that the plastid is essentially cytoplasmic, that it possesses a few "cavities" (probably also cytoplasmic in nature) in which the starch is deposited, that it gives rise upon fixation to a peculiar platework picture, in all likelihood marking particularly active regions within the plastid, and that it gives rise to the apical body in spermatogenesis, we cannot but question Parat's interpretation of the plate-like "active mitochondria," the secretory "vacuoles" and the "diffuse lipoids" of the "zone de Golgi."

If the similarity shown by the plastid to the Golgi zone in structure, function and behavior may be used as evidence for the structure of the Golgi zone, it lends strong support to the interpretation placed upon that cellular element by Morelle, namely, that it is a mass of specialized cytoplasm containing a number of "vacuoles," and that after prolonged treatment with fixing agents a coarse, heavily-staining network appears within the specialized cytoplasmic mass.

I wish to acknowledge my indebtedness to Professor L. W. Sharp for the interest which he has shown in the progress of this work, and for his stimulating and helpful criticisms of the many perplexing questions which have arisen in connection with the study; and to Professor E. B. Wilson for his kindness in loaning me some of the preparations of the late Professor Robert Bowen.

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